AD_____

Award Number: W81XWH-10-1-0917

TITLE: VRP09 Reduction of Corneal Scarring Following Blast and Burn Injuries to Cornea Using siRNAs Targeting TGFb and CTGF

PRINCIPAL INVESTIGATOR: Gregory Schultz, Ph.D.

CONTRACTING ORGANIZATION: University of Florida
Gainesville, Florida 32611-5500

REPORT DATE: March 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
March 2013	Annual	27 September 2010 – 26 February 2013

4. TITLE AND SUBTITLE

VRP09 Reduction of Corneal Scarring Following Blast and Burn Injuries to Cornea Using siRNAs Targeting TGFb and CTGF

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0917

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Gregory Schultz

E-Mail: schultzg@ufl.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Florida
Gainesville, Florida 32611-5500

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Blast and burn injuries to the eye caused by explosions during combat or terrorist attacks are devastating injuries, which typically impair vision by excessive corneal scarring. Our goal is to develop a topical therapy that will reduce corneal scarring by selectively reducing expression of TGFb, TGFb receptor-II (TGFBRII), and CTGF genes which cause scarring using the newly discovered effects of small interfering RNAs (siRNA). We designed up to four siRNAs targeting each of the three target genes and measured their ability to knockdown the target mRNAs and proteins using cultures of rabbit corneal fibroblasts (RCF). The most effective siRNAs for each target gene were tested in combinations of two and three siRNAs and a unique triple combination of siRNAs was identified that synergistically knocked down levels of siRNAs for each of the three target genes and of collagen and alpha smooth muscle actin (aSMA) by over 90% with no toxicity of RCF. The triple combination of siRNAs also reduced transformation of RCF into myofibroblasts and reduced migration of RCF. The siRNAs were combined with cationic nanoparticles (NP) and transfected into ex vivo cultured rabbit corneas that had defined central corneal injuries created by excimer laser ablations. The siRNA-NP transfected corneas had significantly lower levels of collagen and aSMA and less haze (scarring). Similarly transfection of rabbit corneas following excimer laser ablation with the triple siRNA-NP significantly reduced mRNAs of the three target genes as well as collagen and aSMA and also reduced corneal scarring in two of three animals. These results indicate that RNA interference targeting key scarring genes is a viable approach to developing therapies to reduce corneal scarring.

15. SUBJECT TERMS
corneal scarring, siRNA, nanoparticles, TGFb, TGFb receptor, CTGF, collagen, smooth muscle actin

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	33	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

	Page
Introduction……..…….…..	**4**
Body……..	**4**
Key Research Accomplishments…….…….	**32**
Reportable Outcomes……	**33**
Conclusion……	**33**
References……	**33**
Appendices……	**33**

INTRODUCTION

Blast and burn injuries to the eye caused by explosions during combat operations or terrorist attacks are devastating injuries, and in eyes that can be saved, the major causes of vision impairment are excessive corneal scarring and neovascularization. Unfortunately, no approved drugs have been shown to improve vision outcome in eyes with these types of corneal injuries. However, decades of clinical experience and laboratory research have shown that the key to improving vision outcome is to improve the quality of corneal wound healing.[(1), (2), (3)] Our overall objective was to develop new drugs that use the emerging technology of RNA-interference (RNAi) to reduce vision impairment following corneal injuries by reducing expression of genes that stimulate formation of corneal scar (corneal haze).[(4)] We reported previously that corneal scarring is primarily up-regulated by the actions of transforming growth factor beta (TGFb), which stimulates corneal cells by binding to the TGFb type II receptor (TGFbRII) and inducing expression of connective tissue growth factor (CTGF).[(5)] CTGF then directly up-regulates synthesis of collagen scar and induces transformation of fibroblasts into myofibroblasts. Our approach was to design and test small interfering RNAs (siRNA) that will selectively reduce the level of expression of these three key proteins that stimulate corneal scar formation, and thereby, reduce vision loss. We focused on siRNAs because they are the most potent and selective of all gene-targeted, oligonucleotide-based drug approaches (better than ribozymes, antisense oligonucleotides (ASO), or microRNAs).[(4)] To accomplish these objectives, we proposed three specific aims. First, we proposed to design and test siRNAs that selectively target the mRNAs of each of the three target genes, TGFb, TGFbRII and CTGF, using cultures of rabbit corneal fibroblasts (RCF). We then would test the optimal siRNA for each gene when formulated into double and triple combinations using cultures of RCF with the objective to obtain a synergistic, maximum knock-down of collagen and alpha smooth muscle actin (aSMA) synthesis. Second, we proposed to develop an advanced *ex vivo* organ culture system using viable explants of rabbit corneas, and assess the effect of the most effective triple siRNA combination for reduction of target genes, collagen and alpha smooth muscle actin (aSMA) in rabbit corneas following excimer laser ablation. Critical for this second specific aim was the development of an effective and simple method to transfect cells in corneal tissue. Our proposed strategy was to use siRNAs that are complexed with cationic nanoparticles (NP). Third, we proposed to assess the effectiveness of the triple siRNA in the *in vivo* rabbit model of corneal scarring model using excimer laser ablation to create the simulated blast injury to corneas and measure the reduction of corneal scarring (haze). Successful completion of these three specific aims would help establish if siRNAs are a good option to develop gene-targeted therapies to reduce corneal scarring following blast and burn injuries to corneal tissue.

BODY

We accomplished all the primary objectives for this project. The results are sufficiently impressive in cell cultures, *ex vivo* organ culture model and the standard *in vivo* rabbit of corneal scarring to justify the next phase of developing a triple combination of siRNAs as an effective antiscarring drug therapy for corneal blast and burn injuries.

In the first year of the two year research project, we established primary cultures of RCF that we then used to test the siRNAs. We designed and tested at least three siRNAs at that target each of the three target gene mRNAs (TGFb, TGFbRII and CTGF) at multiple concentrations in the RCF cultures. We identified at least one siRNA that knocked-down the level of mRNA at least 70% for each of the target genes in the cultures of RCF. These results were confirmed by measuring the levels of the target proteins in the medium and in extracts of the RCF. We then combined the optimal siRNA for each of the target genes in pairs of two siRNAs and tested the effect on knock-down of target mRNAs. Finally, we formulated a triple combination of siRNAs targeting each the three target genes based on the results from tests of the single and double combination of siRNAs, and tested its ability to knock-down levels of each of the three target genes mRNAs and proteins, and most importantly, to knock-down the level of type-I collagen mRNA in cultures of RCF. We achieved a tremendously positive result, with the triple combination of siRNAs reducing the level of type-I collagen mRNA by 97%, without any general toxic effect on the RCF. This was a very significant accomplishment as shown by the extensive data below.

Objective 1a. Establish primary cultures of rabbit corneal fibroblasts for use in assessing effects of siRNAs on expression of the three target genes.

We successfully established cultures of rabbit corneal fibroblasts using a procedure we described previously in which fresh rabbit corneas were obtained and rinsed extensively to remove bacteria and fungi then chopped into small pieces ~1 mm^3 and placed into T-25 culture flasks with medium containing 20% fetal calf serum. After ~1 week the fibroblasts migrate from the corneal blocks and proliferate. When initial confluency is reached the cultures are split with trypsin/EDTA and seeded into 48 well or 96 well culture plates for use in testing the siRNAs.

Objective 1b. Optimize conditions for transfection of preformed siRNAs into cultures of rabbit corneal fibroblasts.

As shown in Figure 1, several different conditions were evaluated for optimal transfection of preformed siRNAs into low passage cultures of RCF. Initial transfection optimization of siRNAs into rabbit cornea fibroblast (RCF) was performed using the Ambion *Silencer*® siRNA Starter Kit. The kit supplies a GAPDH siRNA and several methods to analyze the concentration of GAPDH after transfection of the GAPDH siRNA. Two different siRNA transfection reagents, siPORT™ *NeoFX*™ Transfection Agent and *Trans*IT-TKO® Transfection Reagent, were used in different quantities to determine which transfection conditions were best suited for RCF transfection. The siPORT™ *NeoFX*™ Transfection Agent

produced the greatest knockdown of GAPDH at approximately 17% when using 0.5 ul of the siPORT™ *NeoFX*™ Transfection Agent. Based on these results, we routinely used 0.5 ul of siPORT™ *NeoFX*™ Transfection Agent to transfect the RCF. Low levels of CTGF, TGF-B1 and TGF-B2R were found in the RCF. Our laboratory has also optimized the stimulation of these cells to produce a greater of amount of these growth factors by adding low levels of estradiol to increase TGFb and TGFbRII mRNAs, and adding low levels of TGFb1 to up-regulate CTGF mRNA. This stimulation simulates the up regulation of CTGF, TGF-B1 and TGFbRII in the cornea after an injury.

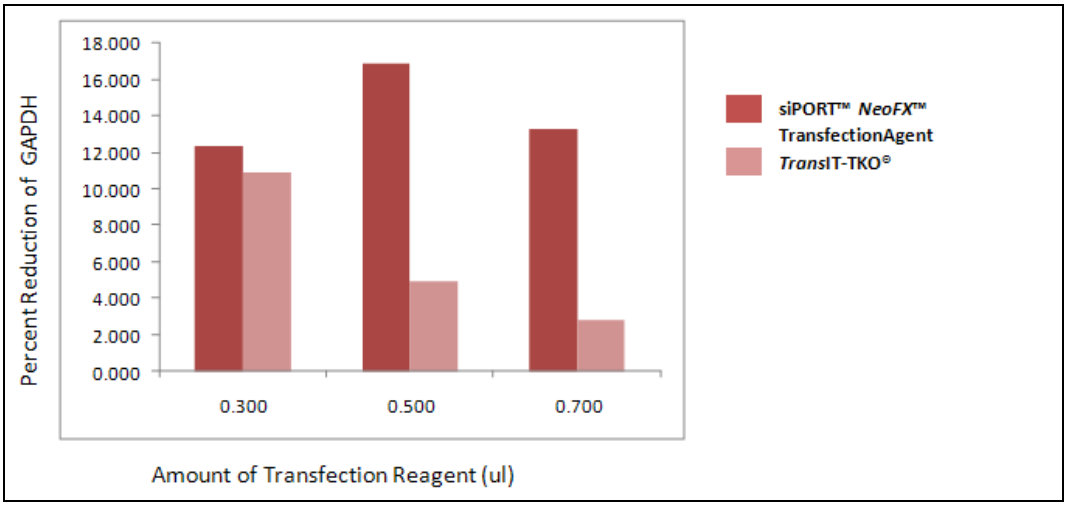


Figure 1. *Optimization of conditions to transfect cultures of Rabbit Corneal Fibroblasts with siRNAs.*

Objective 1c. Design up to four siRNA oligonucleotides (22mers) that selectively target mRNAs for each of three key genes that regulate corneal scarring: TGFb, TGFbRII, and CTGF.

As shown in Table 1, below, we designed siRNAs for all tree target mRNAs using algorithms that were provided by commercial companies and that are available through free software programs. These siRNAs have all been ordered from the manufactures indicated and will be delivered in the next few weeks.

Table 1		**siRNA sequences**		
Target TGFb1	**Sense/Antisense**	**Production_Sequence**	**Start**	
	s	CCAACAUGAUCGUGCGCUCdTdT	305	Sigma & Dharmacon
	a	GAGCGCACGAUCAUGUUGGdTdT	305	
	s	GAGCAGCUGUCCAACAUGAdTdT	295	Sigma & Dharmacon
	a	UCAUGUUGGACAGCUGCUCdTdT	295	
	s	GCAGCUGUCCAACAUGAUCdTdT	297	Sigma & Dharmacon
	a	GAUCAUGUUGGACAGCUGCdTdT	297	
	s	CUACUGCUUCAGCUCCACAUU	13	Ambion/Dharmacon
	a	UGUGGAGCUGAAGCAGUAGUU	13	
Target TGFbRII	**Sense/Antisense**	**Production_Sequence**	**Start**	
	s	GGAAAGAACATGTGAGCAA	7177	Ambion - 7171
	a			
	s	CGACAGGACTATAAAGATA	7313	Ambion - 7318
	a			
	s	CAAACTACCTACAGAGATT	3589	Ambion - 3590
	a			
	s	CAACTAGAATGCAGTGAAA	5033	Ambion - 5034
Target CTGF	**Sense/Antisense**	**Production_Sequence**	**Start**	
	s	AAGCTGACCTGGAAGAGAA	752	Ambion/Dharmacon
	a			
	s	AAGAAGAGCATGATGTTCA	964	AmbionDharmacon
	a			
	s	AAGAAGGGCAAGAAGTGCA	775	Ambion/Dharmacon
	a			
	s	TGGAAGAGAACATTAAGAA	761	Ambion - 764
	a			

Objective 1d. Design and test Q-RT-PCR primers for each of three key genes that regulate corneal scarring: TGFb, TGFbRII, and CTGF and the control house keeping gene GAPDH.

As shown in Table 2, we also designed and ordered primers for the three genes that will be used in Q-RT-PCR assays to measure the levels of the mRNAs in cultures of cells treated with the siRNAs as described below and in rabbit corneas following excimer ablation (blast injury) and thermal injury (burn injury).

TABLE 2		RT-PCR Primers						
CTGF								
Name	Sequence	Start Posit	Strand	Length	Primer Tm	Purity	Modification	Scale
Primer Set 1: Amplicon Size = 82								
AB217855.1_L1	AGGAGTGGGTGTGTGATGAG	300	forward	20	58.51	GAP		0.05 umol
AB217855.1_R1	CCAAATGTGTCTTCCAGTCG	362	reverse	20	59.13	GAP		0.05 umol
AB217855.1_P1	ACCACACCGTGGTTGGCCCT	327	forward	20	69.25	HPLC	5'Fam - 3'Tamra	0.05 umol
TGF B1								
Primer Set 2: Amplicon Size = 86								
AF000133.1_L1	CCTGTACAACCAGCACAACC	189	forward	20	59.06	GAP		0.05 umol
AF000133.1_R1	CGTAGTACACGATGGGCAGT	255	reverse	20	58.68	GAP		0.05 umol
AF000133.1_P1	CTCCAGCGCCTGTGGCACAC	233	reverse	20	69.92	HPLC	5'Fam - 3'Tamra	0.05 umol
TGFBR2								
Primer Set 1: Amplicon Size = 109								
BD061291.1_L1	CGTCGAGACTCCATCTCAAA	4665	forward	20	58.96	GAP		0.05 umol
BD061291.1_R1	AAACAGCCCACAAATGTCAA	4754	reverse	20	59.02	GAP		0.05 umol
BD061291.1_P1	TCAGCTTTGCACAAGGGCCCT	4713	reverse	21	68.48	HPLC	5'Fam - 3'Tamra	0.05 umol
GAPDH								
Primer Set 2: Amplicon Size = 94								
NM_001082253.1_L1	GAGACACGATGGTGAAGGTC	67	forward	20	58.05	GAP		0.05 umol
NM_001082253.1_R1	ACAACATCCACTTTGCCAGA	141	reverse	20	59.14	GAP		0.05 umol
NM_001082253.1_P1	CCAATGCGGCCAAATCCGTT	93	reverse	20	69.17	HPLC	5'Fam - 3'Tamra	0.05 umol

Objective 1e. Test up to four siRNA oligonucleotides (22mers) that selectively target mRNA for TGFb1 in low passage cultures of rabbit corneal fibroblasts for knockdown of TGFb1 protein and mRNA.

As shown in Figure 2, below, three different siRNAs targeting TGFb1 mRNA were tested for knockdown of TGFb1 protein using the optimized transfection conditions that were established in experiments performed during the first quarter of the grant. Two of the siRNAs, siRNA-1 and siRNA2 at 15 nM, 30 nM and 60 nM concentrations were very effective in reducing levels of TGFb1 protein in the conditioned medium. Similarly, siRNA-1 and siRNA-2 at concentration of 15 nM were both effective in knocking down TGFb1 protein in extracts of the rabbit corneal fibroblast cell cultures. In contrast, siRNA3 was not as effective as the other two siRNAs

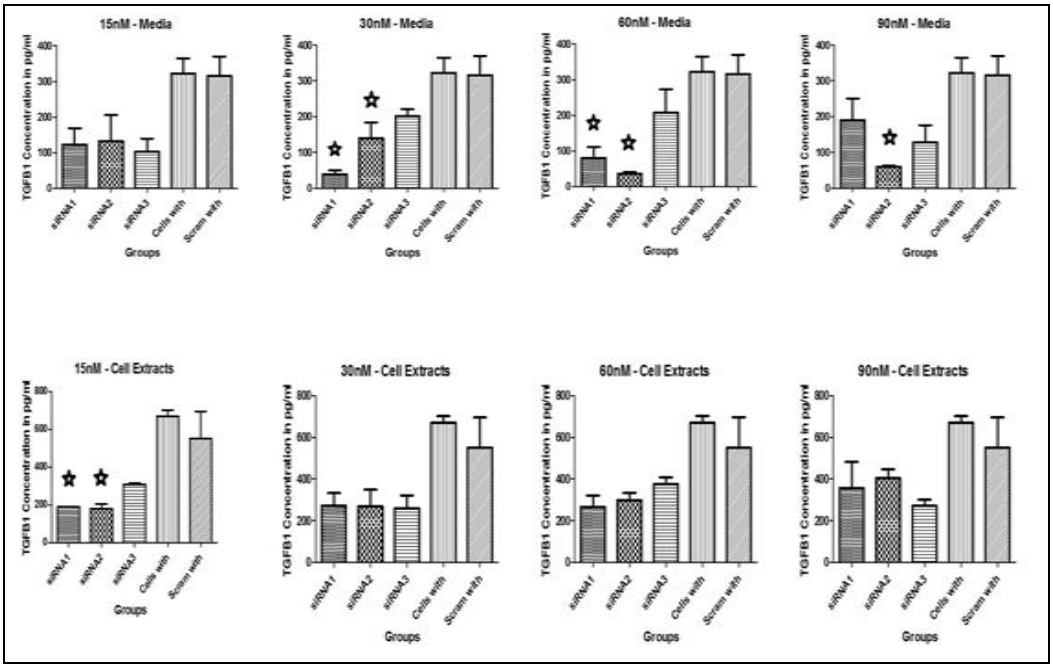


Figure 2. siRNA Knockdown of TGFb1 Protein. *Levels of TGFb1 protein were measured using ELISA in conditioned media and in extracts of low passage rabbit corneal fibroblasts transduced with increasing concentrations of three different siRNAs targeting TGFb1 mRNA. Both siRNA-1 and siRNA-2 significantly reduced levels of TGFb1 protein in conditioned media and cell extracts at low concentrations (15 to 30 nM).*

As shown in Figure 3, siRNA-1 and siRNA 2 met the go/no go cutoff of reducing TGFb1 protein by 80% (arrows) and so were selected to move to the next stage of drug development in testing double and triple combinations of siRNAs. The siRNA3 did not achieve 80% reduction so it was not further evaluated.

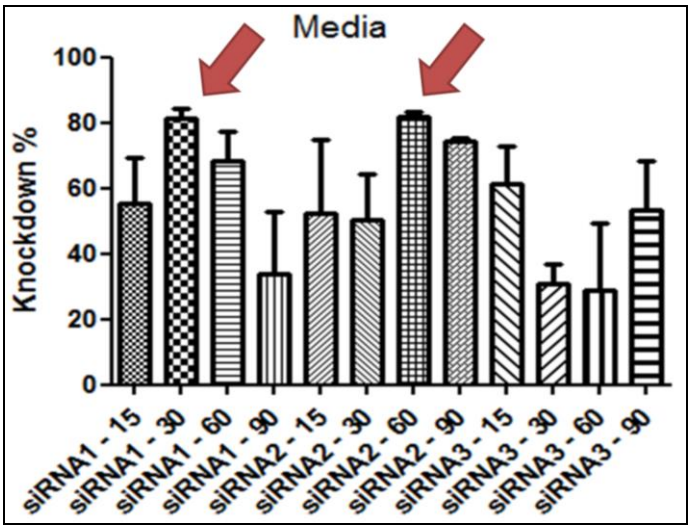


Figure 3*. Percent Knockdown of TGFb1 Protein by siRNAs in Culture Medium of Rabbit Corneal Fibroblasts*.

We also tested the siRNAs for knockdown of TGFb1 mRNA in cultures of rabbit corneal fibroblasts using Q-RT-PCR assays. As shown in Figure 4, levels of mRNA for TGFb1 was reduced by >90% in cultures of cells treated with all three of the siRNAs. In contrast, cells transfected with the scrambled siRNA control showed no knockdown of TGFb1mRNA.

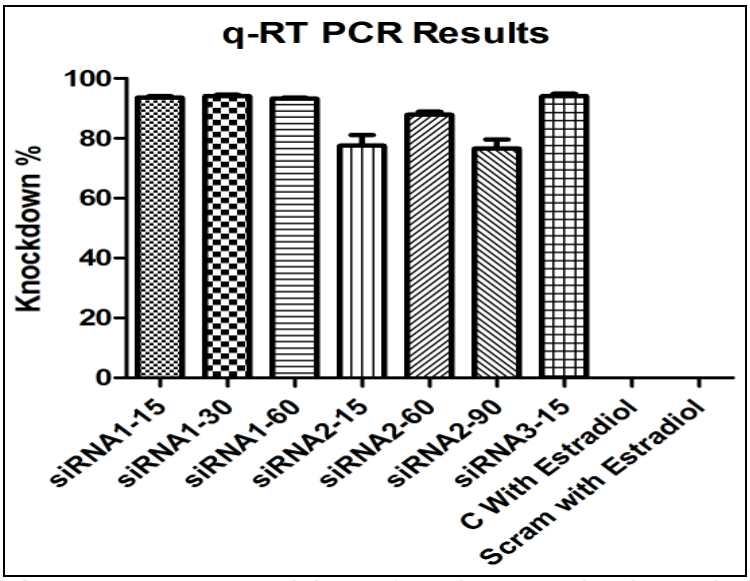


Figure 4. *Percent Knockdown of TGFb1 mRNA by siRNAs in Cultures of Rabbit Corneal Fibroblasts.*

<u>Objective 1f. Test up to four siRNA oligonucleotides (22mers) that selectively target mRNA for CTGF in low passage cultures of rabbit corneal fibroblasts for knockdown of CTGF protein and mRNA.</u>

Similar experiments were performed to test the knockdown of three siRNAs targeting CTGF mRNA and protein in cultures of rabbit corneal fibroblasts. As shown in Figure 5 below, only siRNA3 at concentration of 60 nM significantly reduced levels of CTGF protein in conditioned media of cultures of rabbit corneal fibroblasts that were stimulated by TGFb1 (which induces CTGF mRNA and protein).

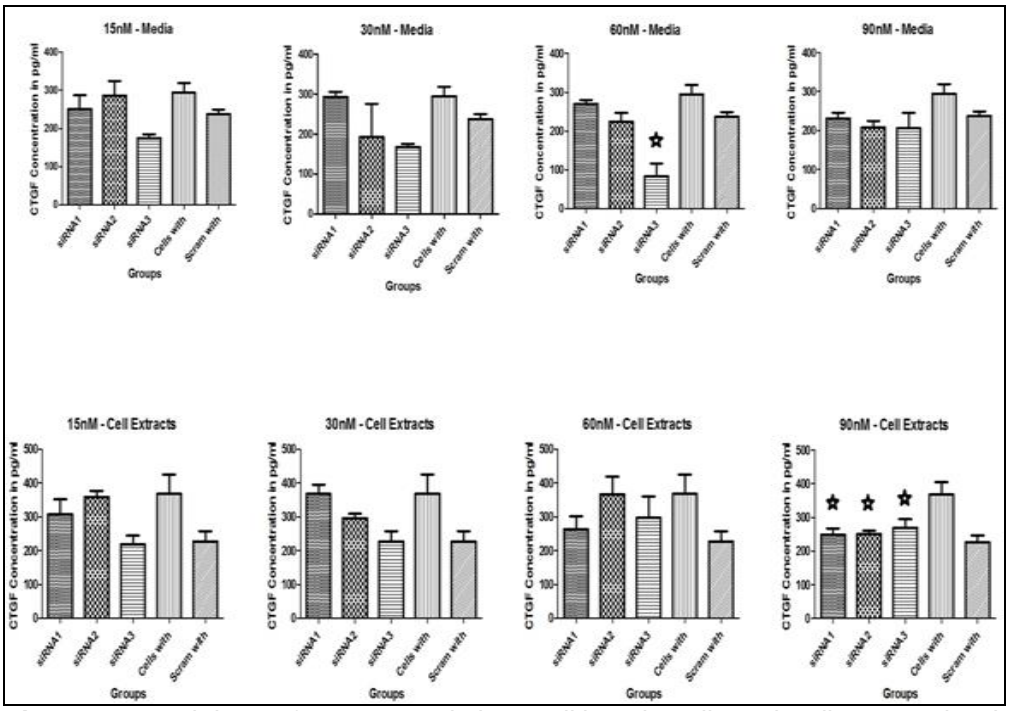


Figure 5. *Knockdown of CTGF Protein in Conditioned Media and Cell Extracts by siRNAs.*

As shown in Figure 6 below, siRNA3 targeting CTGF mRNA produced the best percent knockdown of CTGF protein. However, the level of knockdown was ~75%, which was slightly below the go/no threshold level that was set at 80%.

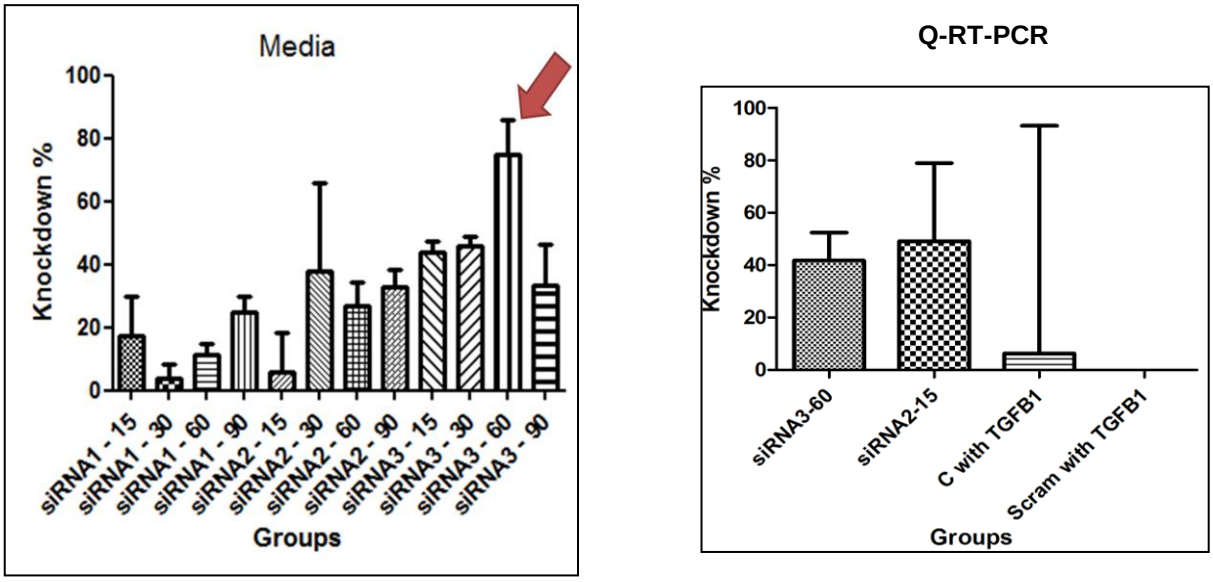


Figure 6. *Percent Knockdown of CTGF Protein and mRNA by siRNAs Targeting CTGF mRNA in Cultures of Rabbit Corneal Fibroblasts.*

<u>Objective 1g. Test two additional siRNA oligonucleotides (22mers) that selectively target mRNA for CTGF in low passage cultures of rabbit corneal fibroblasts for knockdown of TGFb1 protein and mRNA.</u>

In previous experiments, we identified one siRNA targeting CTGF mRNA that knocked down CTGF protein ~60% so we designed two new siRNAs targeting CTGF mRNA. As shown in Figure 7, two additional siRNAs targeting CTGF mRNA were tested for knockdown of CTGF protein using the optimized transfection conditions that were established in previous experiments. Both of the siRNAs (siRNA-1 and siRNA-2) produced significant knockdown of CTGF protein: siRNA-1 reduced CTGF protein the cell extract ~70% when added at 15 nM concentration and 60 nM; siRNA-2 added at 15 nM concentration reduced levels of CTGF protein ~80% in the conditioned medium of the estrogen stimulated rabbit corneal

fibroblasts; siRNA-2 added at 60 nM also showed good knockdown of CTGF protein (~65%) in both conditioned medium and cell extract. Based on these results, we focused on siRNA-2 targeting CTGF.

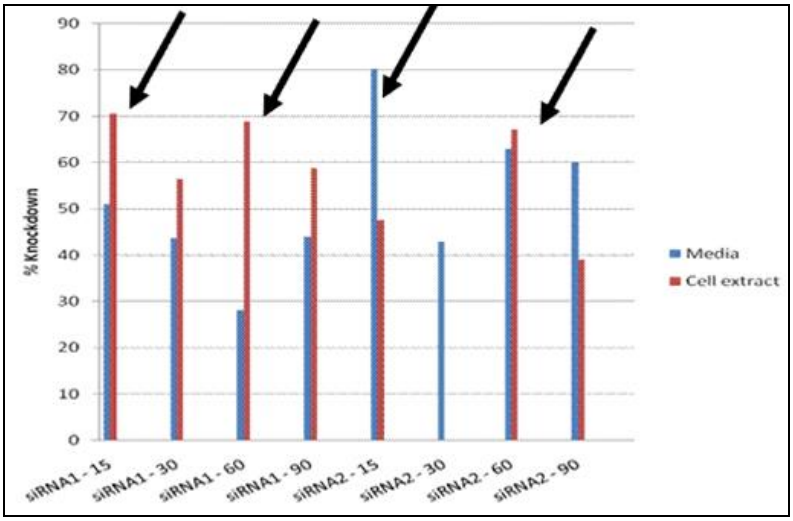


Figure 7. *Both siRNA-1 and siRNA-2 targeting CTGF mRNA significantly knocked down levels of CTGF protein in cultures of rabbit corneal fibroblasts stimulated by TGFb1.*

Objective 1h. Test siRNA oligonucleotides (22mers) that selectively target mRNA and protein for TGFbRII in low passage cultures of rabbit corneal fibroblasts.

Similar experiments were performed to test the knockdown of three siRNAs targeting TGFbRII mRNA and protein in cultures of rabbit corneal fibroblasts. As shown in Figure 8 below, both of the siRNAs achieved >80% knockdown of TGFbRII protein in conditioned media at concentrations of 15nM and 30nM in cultures of rabbit corneal fibroblasts that were stimulated by estrogen, which induces TGFbRII mRNA and protein.

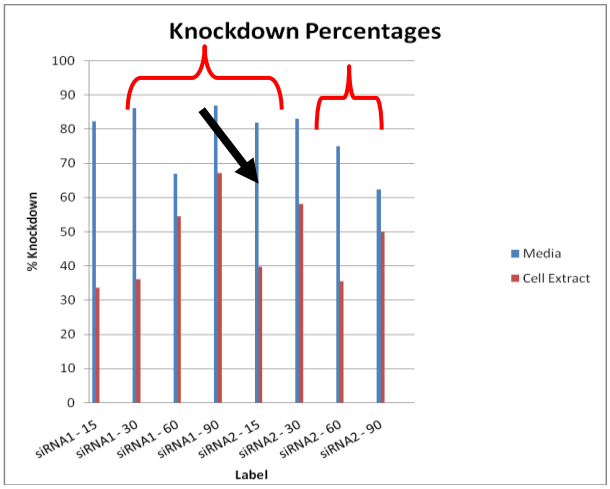


Figure 8. Individual TGFBR2 siRNA knockdown percentages.

Table 3. Nucleotide sequences of siRNAs targeting CTGF and TGFbRII

Growth Factor	siRNA sequence
TGFbR2	GCAGAGAACTTGAAAGCAT
TGFbR2	CCATATGCGGTGTGAAATA
CTGF	GUGAUGAGCCCAAGGACCA
CTGF	GCGAGGAGUGGGUGUGUGA

Objective 1g. Test the combination of two siRNA oligonucleotides targeting two separate target genes on the level of TGFb1 protein and mRNA in low passage cultures of rabbit corneal fibroblasts.

One of our key hypotheses is that the combination of two or more siRNAs targeting multiple genes in the TGFb-TGFbRII-CTGF system pathways would have the optimal knockdown of a target gene, like TGFb1. To test this hypothesis, we first needed to identify the siRNAs that had optimal knockdown of each of the separate genes, which we completed during the third quarter of the project.

The results of the first of several experiments combining siRNAs targeting different genes is shown in Figures 9 and 10. The two optimal siRNAs targeting TGFb1 (designated T1 and T2) and the two most effective siRNAs targeting TGFbRII (designated R1 and R2) were added alone (at 60 nM concentration) and in combinations (each at 30 nM for a final combined concentration of 60 nM) to cultures of rabbit corneal fibroblasts and the level of TGFb1 protein were measured using ELISA for TGFb1. Controls included adding each of the four siRNAs separately with the negative control scrambled ASO (designated NC). The combination of siRNAs T1 and R1 produced a knockdown of ~80% of TGFb1 protein in the conditioned medium of rabbit corneal fibroblasts. This was ~20% greater total knockdown than was generated by the effects of either siRNA added separately. Thus, these data supported our hypothesis that the combination of two siRNAs targeting two different genes, TGFb1 and TGFbRII, would produce significantly better knockdown than ether siRNA alone. The effect of the combination of T1+R1 siRNAs was not synergistic or additive, but the knockdown was significantly better than was achieved with either siRNA alone.

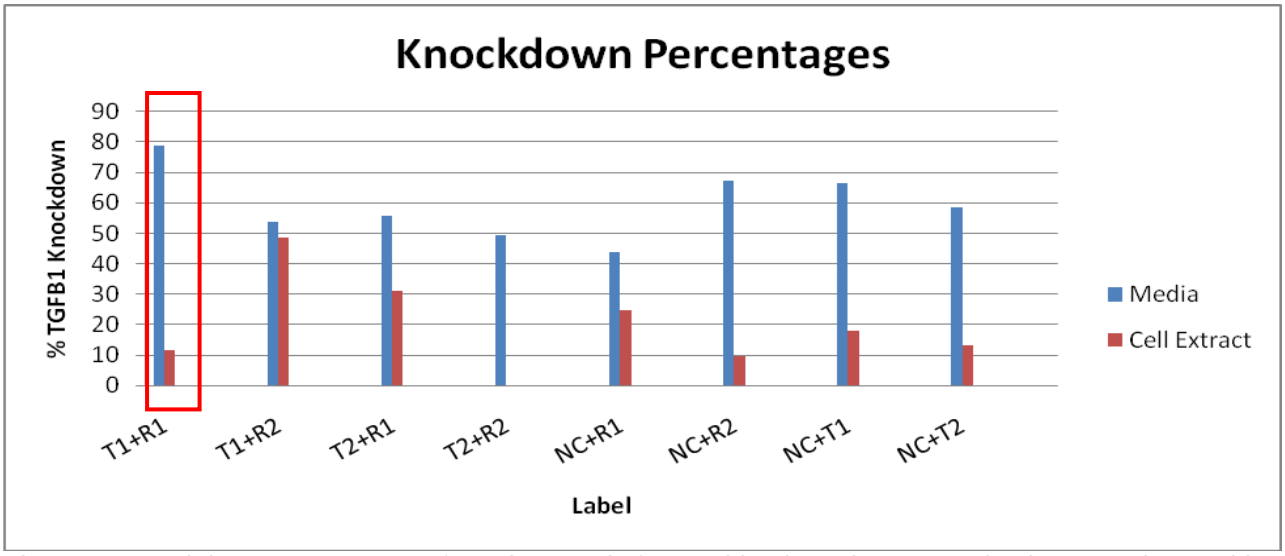


Figure 9. Knockdown percentages of TGFb1 protein for combined TGFbR2 + TGFb1siRNAs. The combination of T1+R1 siRNAs was more effective than either siRNA alone and more effective than other combinations of siRNAs.

Since the percent knockdown of TGFb1 protein does not provide information on the absolute levels of TGFb1 protein in the conditioned medium and in the cell extract, we also re-plotted the results for the combination of two siRNAs on TGFb1 using the actual amount of TGFb1 protein in the 96 well plate (the volume of conditioned medium and cell extract were both 150 ul so concentration is the same as total pg/well). As shown in Figure 4, the combination of T1+R1 siRNAs reduced the total amount of TGFb1 protein to levels that were essentially equal to unstimulated cells. In other words, near baseline levels of TGFb1 protein.

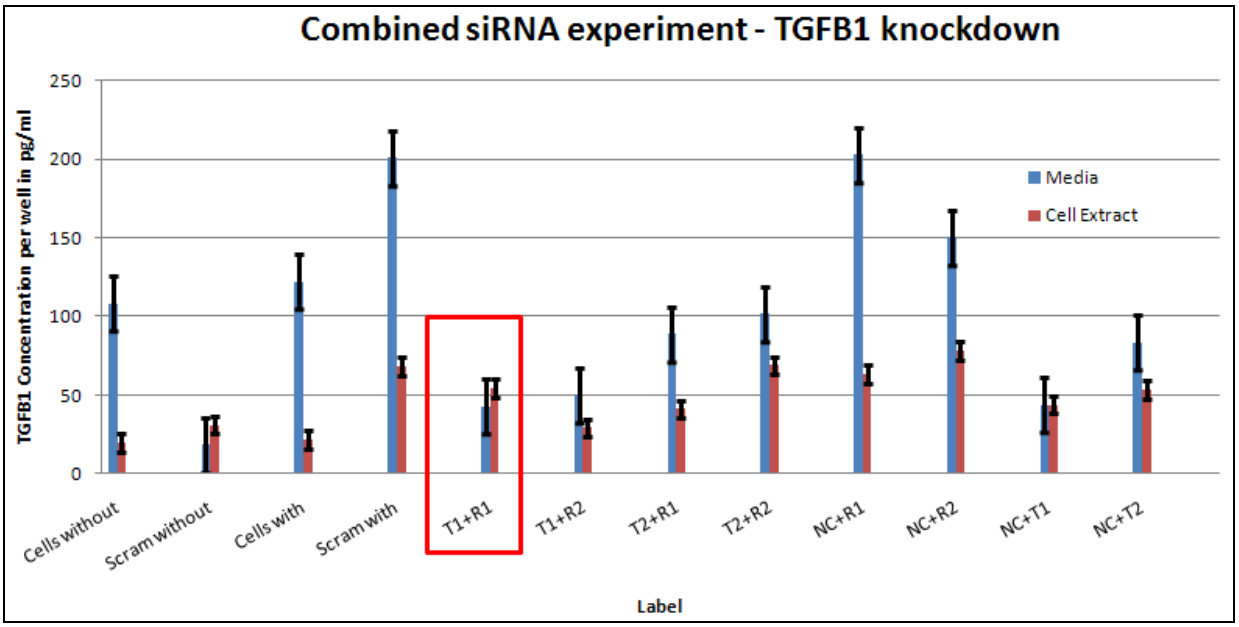


Figure 10. Concentration of TGFb1 protein in cells and medium of rabbit corneal fibroblasts treated with single or combined TGFbR2 + TGFb1siRNAs. The combination of T1+R1 siRNAs was more effective than either siRNA alone and more effective than other combinations of siRNAs.

The addition of siRNAs alone (at 60 nM) or in combinations (each siRNA at 30 nM) was tested for toxic effect on low passage cultures of rabbit corneal fibroblasts. As shown in Figure 11, cell viability was not reduced by addition of single siRNAs or combinations of siRNAs, at final concentrations of 60 nM siRNA oligonucleotides. Thus, the reduction in levels of target proteins and mRNAs was not due to general toxicity due to off-target effects of the siRNAs. This is an important control to perform to understand the knockdown effects and to perform before we begin tests in rabbit corneas following excimer laser ablation.

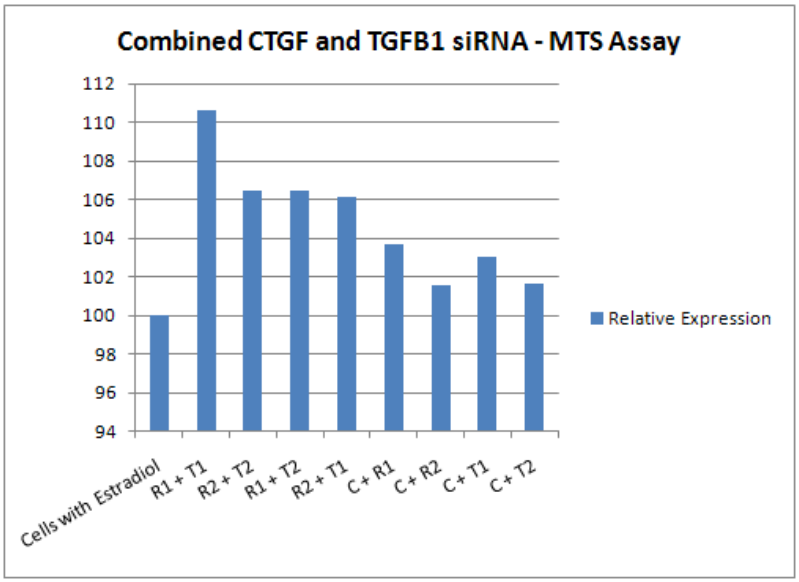


Figure 11. Assessment of cytotoxicity of siRNAs on rabbit corneal fibroblasts. Addition of single siRNAs at 60nM or combinations of siRNAs at 30 nM each did not reduce cell viability as measured by the MTS assay.

Similar experiments were performed using double combinations of siRNAs to TGFb and TGFbRII, and double combinations of TGFb and CTGF siRNAs as shown below in Figures 12, 13 and 14. In all these double combination experiments, optimal combinations of siRNAs were identified for knock down of TGFb, TGFbRII and CTGF mRNAs and proteins.

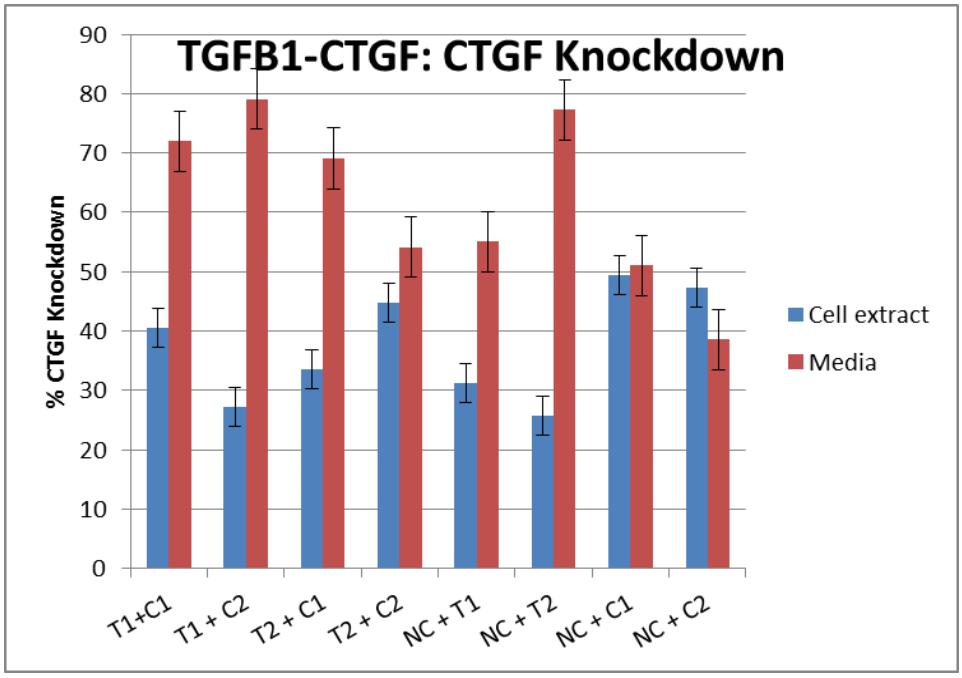


Figure 12. Protein level CTGF knockdown measured by ELISA. The CTGF knockdown % of TGFB1 and CTGF combination siRNA sequences (30nM total concentration) is compared with individual siRNA sequences (combined with scrambled to get 30nM total concentration).

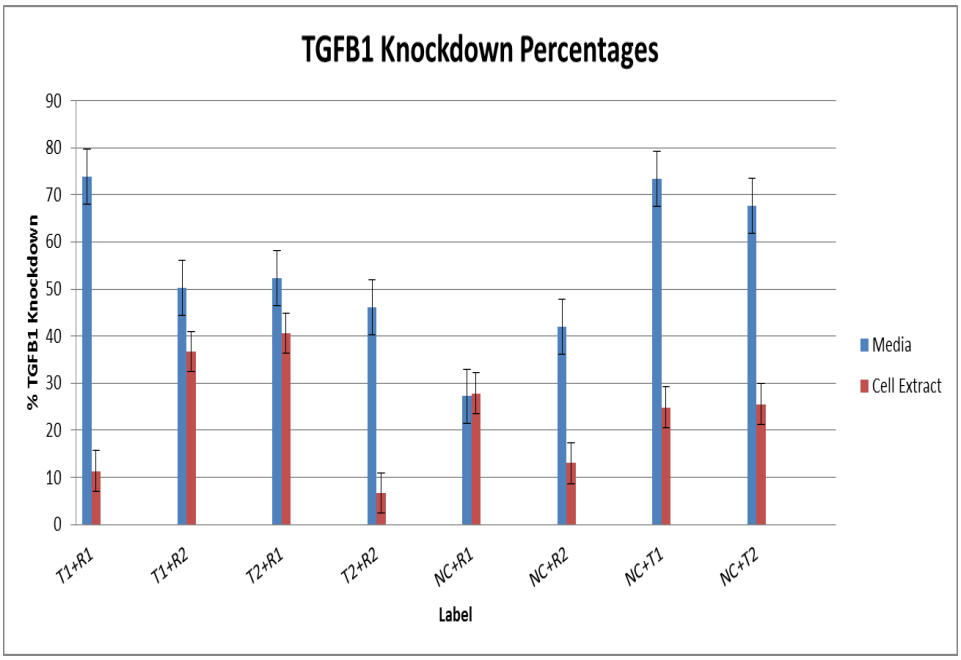


Fig 13. Protein level TGFB1 knockdown measured by ELISA. TheTGFB1 knockdown % of TGFB1 and TGFBR2 combination siRNA sequences (30nM total concentration) is compared with their respective individual siRNA sequences (combined with scrambled to get 30nM total concentration).

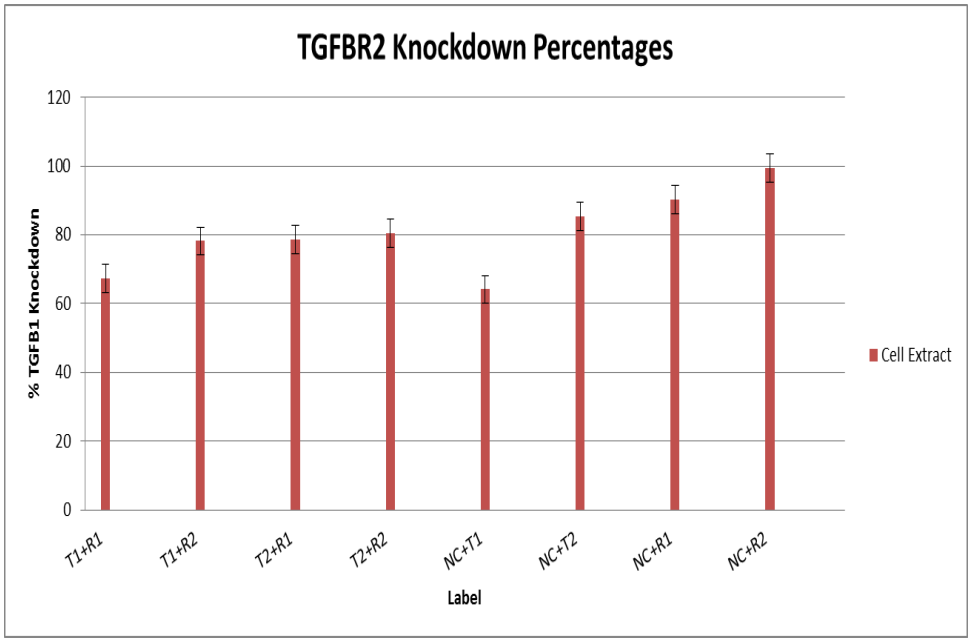


Fig 14. Protein level TGFBR2 knockdown measured by ELISA. TheTGFBR2 knockdown % of TGFB1 and TGFBR2 combination siRNA sequences (30nM total concentration) is compared with individual siRNA sequences (combined with scrambled to get 30nM total concentration).

Objective 1h. Identify the optimal triple combination of siRNAs targeting TGFb, TGFbRII and CTGF mRNAs based on effects of the combinations of two siRNA in low passage cultures of rabbit corneal fibroblasts for knockdown of collagen type I mRNA

The previous experiments identified the most effective single and double combinations of siRNAs targeting the three target genes. In the final series of *in vitro* experiments, we identified the best theoretical triple combination of siRNAs using the following mathematical equations.

- When two or more drugs are added in combination, one of the following three effects can be observed:
    - Additive (indifferent) effect: the activity of two drugs in combination is equal to the sum (or a partial sum) of their independent activity when studied separately
    - Synergistic effect: the activity of two drugs in combination is greater to the sum of their independent activity when studied separately
    - Antagonistic effect: the activity of two drugs in combination is less to the sum (or a partial sum) of their independent activity when studied separately

In order to construct the best triple combination, we have to analyze the results so that the presence of 1 siRNA sequence does not have an antagonistic effect on the other. The following are the basic equations involved in computing a Combination Index (CI) which would tell us if the sequences are synergistic, antagonistic or additive.

$$f_a = 1/[1 + (D_m/D)^m]$$

fa – is the fraction affected by siRNA seq (1/100 * knockdown %)
D – dose / concentration of the drug
Dm - median-effect dose (e.g., IC50, ED50, or LD50) that inhibits the system under study by 50%
m - coefficient signifying the shape of the dose-effect relationship

Combination Index:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = \frac{(D)_1}{(D_m)_1[f_a/(1-f_a)]^{1/m_1}} + \frac{(D)_2}{(D_m)_2[f_a/(1-f_a)]^{1/m_2}}$$

Where CI < 1 indicate synergism, CI = 1 indicates additive effect, and CI > 1 is antagonism. In the denominator, (Dx) is for D1 “alone” that inhibits a system x%, and (Dx)2 is for D2 “alone” that inhibits a system x%.

Table 4

Drug	CI Values at					
	ED50	ED75	ED90	Dm	m	r
C1	N/A	N/A	N/A	1580.57311	-0.155	0.3
(Not a combination)						
C2	N/A	N/A	N/A	66.64924	0.05718	0.0
(Not a combination)						
R1	N/A	N/A	N/A	44.29044	0.79147	0.9
(Not a combination)						
R2	N/A	N/A	N/A	8539.75283	0.03192	0.0
(Not a combination)						
T1	N/A	N/A	N/A	86.27116	-0.50412	0.9
(Not a combination)						
T2	N/A	N/A	N/A	12.15503	0.88476	0.8
(Not a combination)						
T1C1	0.37864	31.28352	38786	30.97464	6.83312	1
(1:1)						
T1C2	0.75005	3.32399	33.79837	28.20254	7.84783	1
(1:1)						
T2C1	0.03828	0.01758	0.99459	0.46173	-0.36361	1
(1:1)						
T2C2	71.51209	512.33054	4339.85851	735.15832	0.32519	1
(1:1)						
T1R1	0.77998	3.74518	47.70293	22.82672	2.63302	1
(1:1)						
T1R2	0.2399	2.89268	35.23193	20.48946	3.4277	1
(1:1)						
T2R1	2.16929	0.83737	0.32431	20.68972	3.43782	1
(1:1)						
T2R2	1.66514	0.65262	0.25614	20.21109	3.58495	1
(1:1)						

Hence, combining T1-C1-R2 would give us the best effect.

Table 5. Triple combination logic for maximum TGFB1 knockdown:

Drug	CI Values at					
	ED50	ED75	ED90	Dm	m	r
C1	N/A	N/A	N/A	1580.57311	-0.155	0.39475
(Not a combination)						
C2	N/A	N/A	N/A	66.64924	0.05718	0.08759
(Not a combination)						
R1	N/A	N/A	N/A	44.29044	0.79147	0.94924
(Not a combination)						
R2	N/A	N/A	N/A	8539.75283	0.03192	0.06065
(Not a combination)						
T1	N/A	N/A	N/A	86.27116	-0.50412	0.91994
(Not a combination)						
T2	N/A	N/A	N/A	12.15503	0.88476	0.80225
(Not a combination)						
T1C1	0.37864	31.28352	38786	30.97464	6.83312	1
(1:1)						
T1C2	0.75005	3.32399	33.79837	28.20254	7.84783	1
(1:1)						
T2C1	0.03828	0.01758	0.99459	0.46173	-0.36361	1
(1:1)						
T2C2	71.51209	512.33054	4339.85851	735.15832	0.32519	1
(1:1)						
T1R1	0.77998	3.74518	47.70293	22.82672	2.63302	1
(1:1)						
T1R2	0.2399	2.89268	35.23193	20.48946	3.4277	1
(1:1)						
T2R1	2.16929	0.83737	0.32431	20.68972	3.43782	1
(1:1)						
T2R2	1.66514	0.65262	0.25614	20.21109	3.58495	1
(1:1)						

Hence, combining T1-C1-R2 would give us the best effect.

Table 6. Triple combination logic for maximum CTGF knockdown:

Summary table						
Drug	Combination Index Values at					
	ED50	ED75	ED90	Dm	m	r
T1	N/A	N/A	N/A	86.27116	-0.50412	0.91994
(Not a combination)						
T2	N/A	N/A	N/A	12.15503	0.88476	0.80225
(Not a combination)						
C1	N/A	N/A	N/A	1580.573	-0.155	0.39475
(Not a combination)						
C2	N/A	N/A	N/A	66.64924	0.05718	0.08759
(Not a combination)						
R1	N/A	N/A	N/A	44.29044	0.79147	0.94924
(Not a combination)						
R2	N/A	N/A	N/A	8539.753	0.03192	0.06065
(Not a combination)						
C1T1	0.175	1.45446	181.3983	14.31614	-0.51436	1
(1:1)						
C1-T2	1.21267	3.61531	1327.71	14.62755	-0.95457	1
(1:1)						
C2T1	0.36642	0.59505	2.21716	13.77753	-1.2717	1
(1:1)						
C2-T2	0.71327	0.00012	2.42E-08	7.33253	-0.15104	1
(1:1)						
C1-R1	0.56457	3.72344	12058	24.32349	-3.6375	1
(1:1)						
C1-R2	0.01965	4.00818	11836	26.20443	-3.1518	1
(1:1)						
C2-R1	0.51779	0.03272	0.00344	13.77753	-1.2717	1
(1:1)						
C2-R2	0.49416	1.56E-09	4.98E-18	32.68037	-3.1363	1
(1:1)						

Hence, combining T1-C1-R2 would give us the best effect.

Table 7. Triple combination logic for maximum TGFBR2 knockdown:

Summary table						
Drug	Combination Index Values at					
	ED50	ED75	ED90	Dm	m	r
T1	N/A	N/A	N/A	86.27116	-0.50412	0.91994
(Not a combination)						
T2	N/A	N/A	N/A	12.15503	0.88476	0.80225
(Not a combination)						
C1	N/A	N/A	N/A	1580.573	-0.155	0.39475
(Not a combination)						
C2	N/A	N/A	N/A	0.48728	2.17234	0.99769
(Not a combination)						
R1	N/A	N/A	N/A	44.29044	0.79147	0.94924
(Not a combination)						
R2	N/A	N/A	N/A	8539.753	0.03192	0.06065
(Not a combination)						
R1-T1	1.42901	2.72225	13.75632	41.8212	-2.1659	1
(1:1)						
R2-T1	0.44168	3.17428	23.04343	37.72317	-5.5779	1
(1:1)						
R1-T2	8.48091	1.03333	0.12632	80.88714	-1.318	1
(1:1)						
R1-C1	31.95939	2.49621	5944.328	1376.912	-0.41089	1
(1:1)						
R2-C1	0.01775	1.08717	29684	23.67703	6.78655	1
(1:1)						
R1-C2	59.17178	4.24457	9.97099	28.51957	-2.8901	1
(1:1)						
R2-C2	1.52796	0.31762	0.06603	0.74451	-1.0315	1
(1:1)						
R2-T2	3.21598	0.75635	0.17814	39.03471	-5.3786	1
(1:1)						

Hence, combining T1-C1-R2 would give us the best effect.

Table 8. Comparison of the collagen knockdown % of the siRNA triple combination made from the sequences T1, C1 and R2 to their respective individual sequences.

T1C1R2 effect on Collagen knockdown

	T1 alone %	C1 alone %	R2 alone %				T1C1R2 %
10nM	48.8	98.9	86			15nM	18.1
15nM	0	0	94.32			30nM	83.1
20nM	27.6	85.32	81.8			60nM	97.1
30nM	82.8	70.46	42			90nM	88.04

As shown in Figure 15, the percent knock-down of TGFbRII and CTGF mRNAs by the triple combination of siRNAs was very good at approximately >95% when the three siRNAs were added at 30 nM eack.

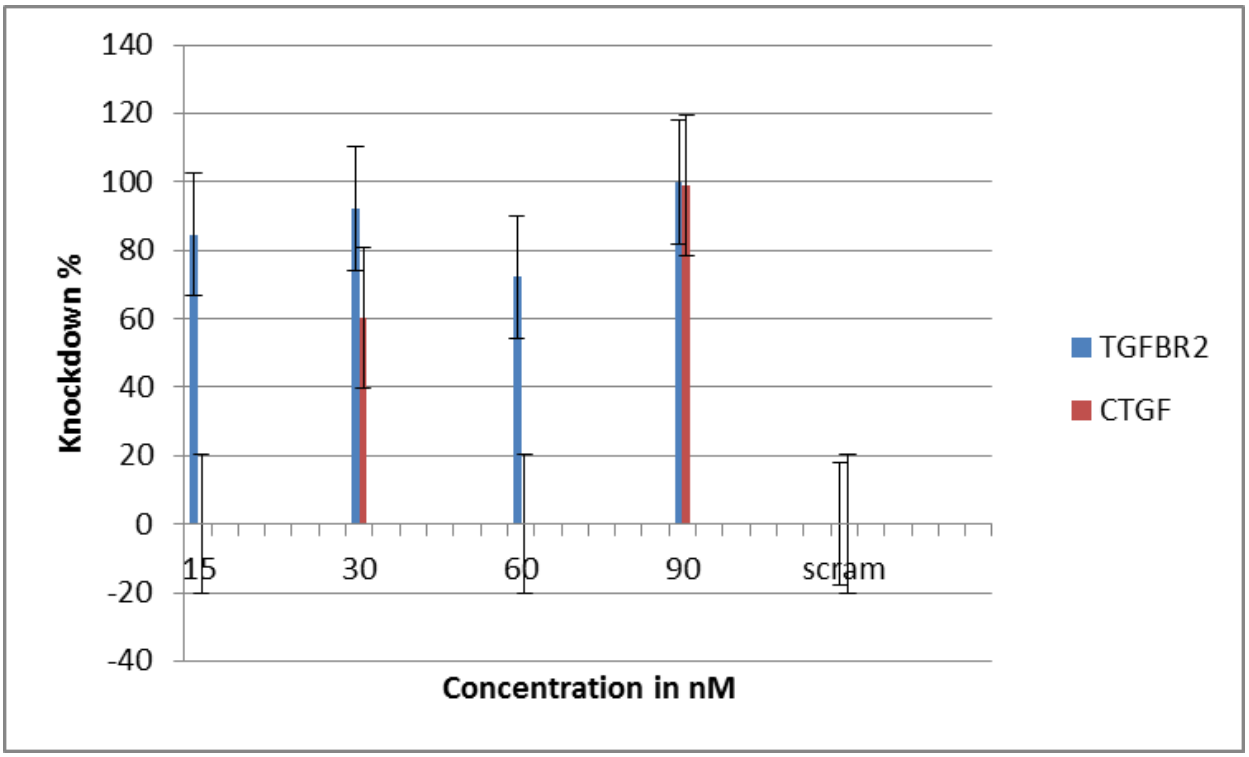


Figure 15. RNA levels of TGFbRII and CTGF knockdown measured by q RT PCR. TGFbRII and CTGF knockdown percent of the triple combination made from siRNA sequencesT1,C1 and R2 are calculated at different total siRNA concentrations.

In Figure 16 shown below, the level of type I collage mRNA knock down by each of the separate siRNAs at 30 nM was 40% for R2 siRNA; was 70% for C1 siRNA; and was 80% for T1 siRNA. Thus, none of the individual siRNAs achieved over 80% knock down alone of the type I collagen mRNA. In marked contrast, as shown in Figure 17, the triple combination of the three siRNAs (T1, R2 and C1) each at 20 nM cause a remarkable 97% knock-down of the mRNA for type I collagen mRNA.

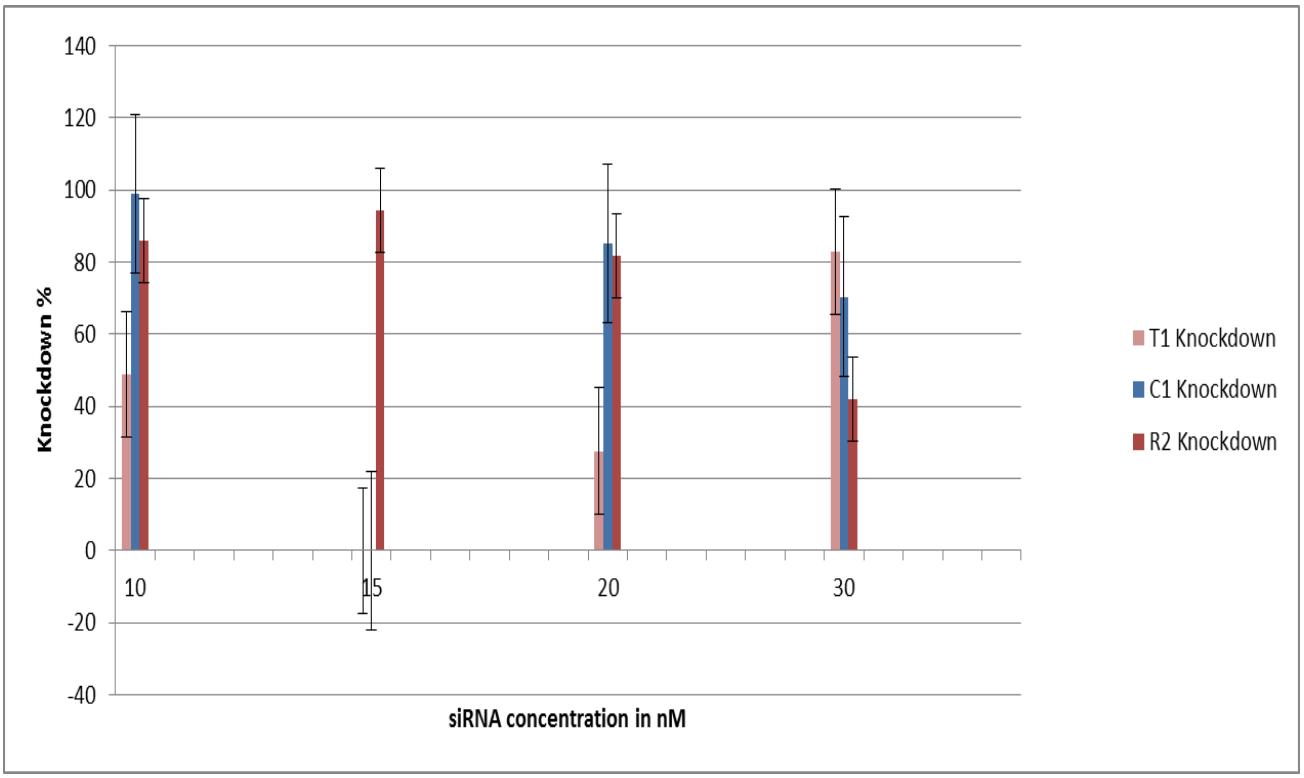


Figure16. RNA level Collagen knockdown measured by q RT PCR. Collagen knockdown percent of the individual siRNA sequencesT1,C1 and R2 are calculated at different total siRNA concentrations.

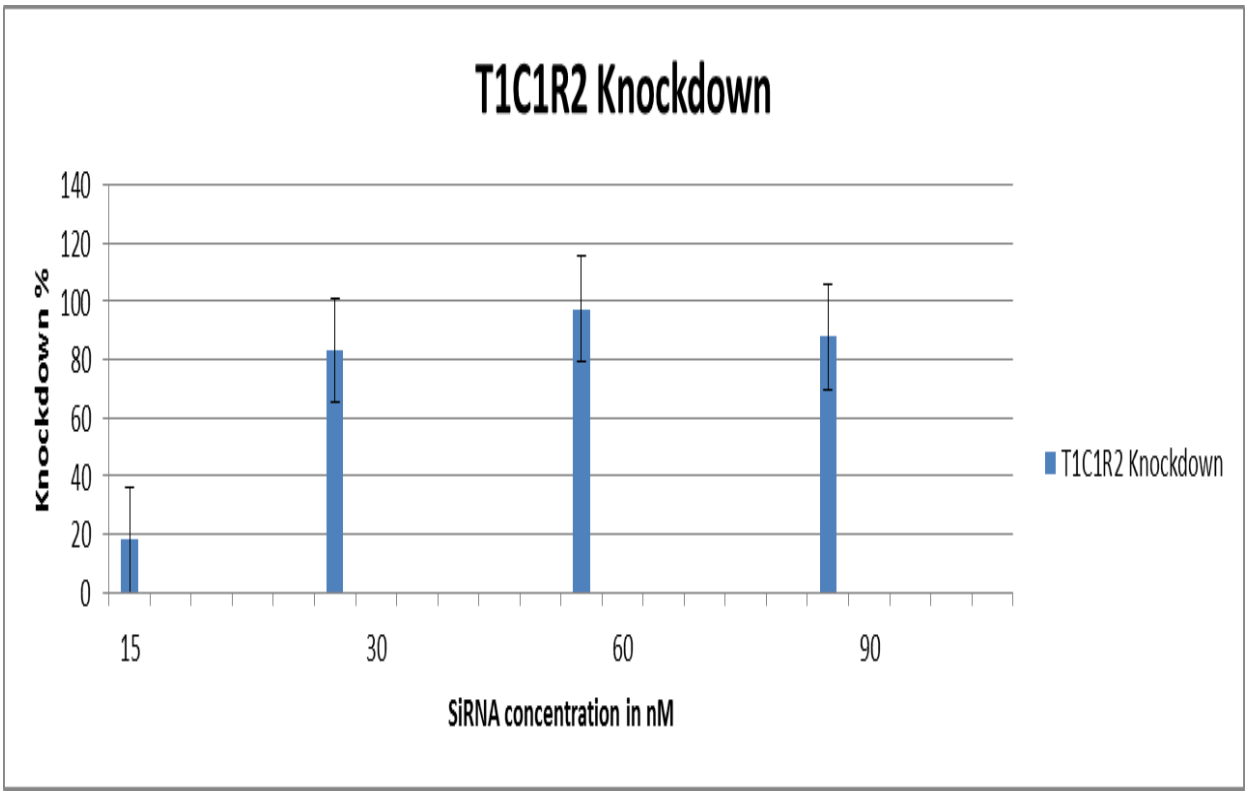


Figure 17. RNA level Collagen knockdown measured by q RT PCR. Collagen knockdown percent of the triple combination made from siRNA sequencesT1,C1 and R2 are calculated at different total siRNA concentrations.

In summary, we successfully identified a triple combination of siRNAs targeting TGFb, TGFbRII, and CTGF mRNAs and achieved a remarkable 97% knockdown of collagen type I mRNA in cultures of corneal fibroblasts when the siRNAs were added at 20 nM concentrations.

Figure 18. **Knockdown of target growth factors by Synergistic Triple combination (T1R2C1)**. Cultures of Rabbit corneal fibroblasts stimulated with TGFB1 and Estradiol were transfected with the triple siRNA combination (15nM, 30nM, 60nM and 90nM) using Mirus transfection reagent. The RNA samples were collected 48 hours after transfection and were analyzed using qRT PCR. On a similar experiment, cells were dosed with increasing concentrations of siRNA triple combination (90nM to 180nM) and cell viability was assessed using the MTS assay. The knockdown percentages of a) TGFB1, b) TGFBR2 and c) CTGF are plotted for the identified synergistic triple combination (T1R2C1). All expressions were normalized to 18S rRNA. The knockdown percentages were calculated with respect to a scrambled siRNA control. d) The percentage of viable rabbit corneal epithelial cells after treating with increasing concentrations of the synergistic triple combination (T1R2C1).

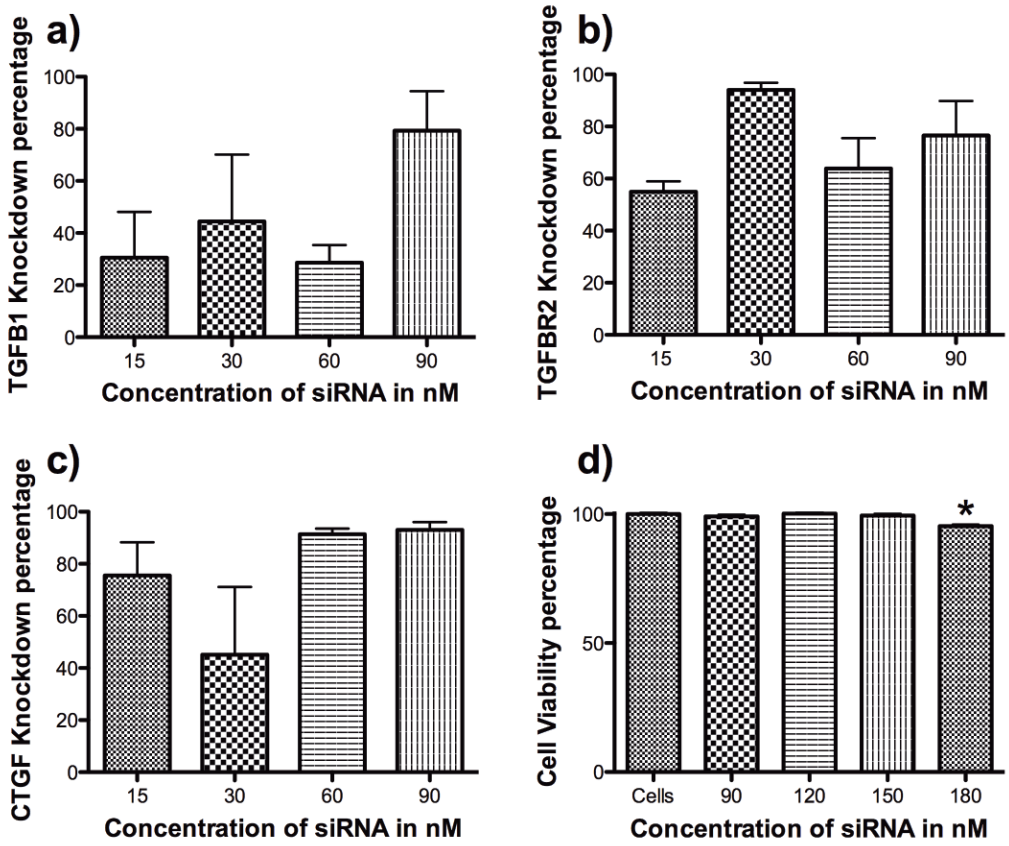


Figure 19. **Reduction of Downstream mediators by the Synergistic Triple combination (T1R2C1).** Cultures of Rabbit

Objective 2a Develop a method to measure amount of corneal haze (scar) that occurs in the rabbit cornea.

As shown in Figure 20 below, corneas injured with a large (6 mm diameter) central ablation created with an excimer laser begin to develop visible haze (scar) beginning about day 6 after injury and the extent of corneal haze continues to increase over the next several days. Control corneas that were not injured with the excimer laser remained clear.

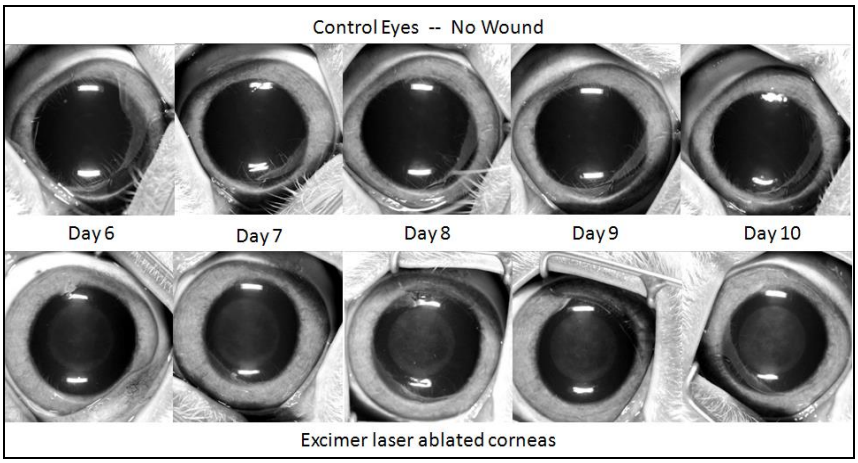


Figure 20. Photographic images of rabbit corneas injured with a large central ablation wound developed progressively more severe corneal haze (scar) while control rabbit corneas remained clear.

As shown in Figure 21, using this standardized photographic technique the digital images were processed by software (Photoshop) and converted into false color maps with integration of total light scatter pixel intensity in the area of the ablation. This method now allows us to quantitatively measure the level of light scatter which is clinically described as haze, and it will be used to assess the effects of the siRNAs on reducing corneal scarring (haze) on rabbit corneas following blast and burn injuries.

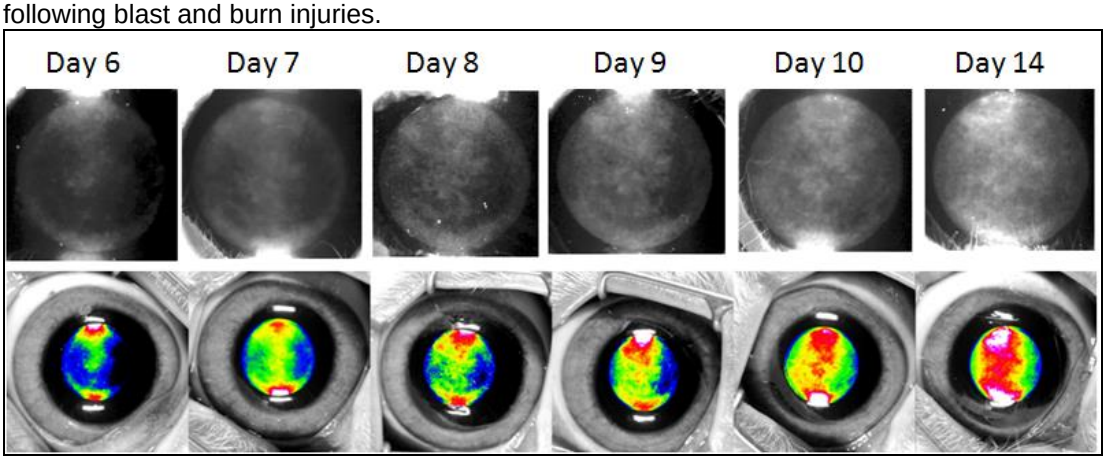


Figure 21. Quantitative analysis of photographic images of rabbit corneas following excimer laser ablation will enable us to assess the effects of the siRNA treatments on reducing corneal haze (scar) following blast and burn injuries to rabbit corneas.

Objective 2b Develop an *ex vivo* organ culture model using viable rabbit corneas to assess the effects of the triple siRNA formulation on reducing target genes and corneal haze (scarring).

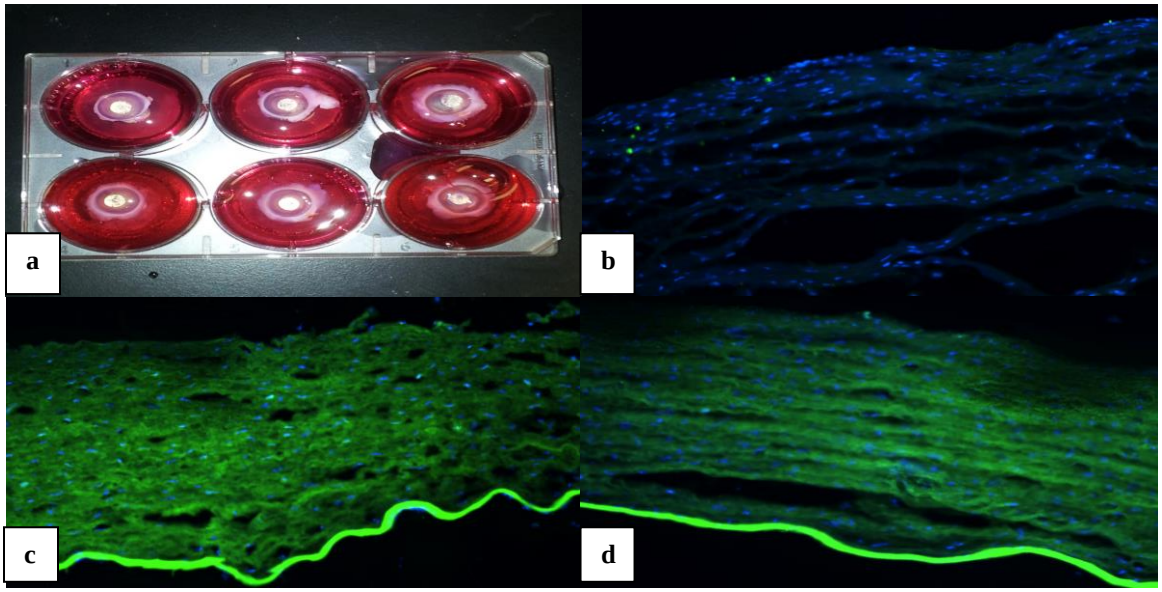
Objective 2b. Assess the effectiveness of nanoparticles complexed with siRNAs to deliver the siRNAs to corneal cells in rabbit eyes.

We had previously assess the effectiveness of iontophoretic delivery of small oligonucleotide sequences (antisense oligonucleotides, ASOs) into rabbit corneal cells and found that technique was very effective in delivering ASOs into the stroma and even into the endothelial cell layer. However, that technique requires the use of a specialized iontophoresis cell that attaches to the cornea surface and a low voltage DC power supply to deliver the electrical current that moves the charged ASOs into the cornea.

To simplify the delivery approach, we assessed the effect of nanoparticles complexed with siRNAs for delivery to rabbit corneal cells. As shown in Figure 22, we generated nanocarrier particles complexed with siGLO which is a fluorescein-labeled siRNA that is a surrogate for our siRNAs targeting the three target genes. The siGLO complexed to nanocarrier particles that we generated with a commercially available kit of biodegradable polymers were tested in vitro using fresh rabbit globes that were ablated by excimer laser to simulate a corneal blast or burn wound. The basic in vitro test model consisted of topically applying the siGLO reporter oligo complexed to nanocarrier particles for 1 minute to the ablated area with the aid of a 14 mm vacuum trephine collar attached to the cornea. The corneas were placed in 6-well culture plates and maintained for several hours to assess uptake of the fluorescein-labeled siRNA nanocarrier particles (panel A). As seen in panel B, the uptake of siGLO when applied to the corneas without nanocarrier particles was very minimal and

typically limited to the upper 1/3 of the corneal stroma. In marked contrast, application of the siGLO complexed to nanocarrier particles (panel C) after brief pretreatment of the corneas with a very dilute EDTA solution to increase permeability of the epithelial cell layer, or in panel D without EDTA pretreatment showed extensive fluorescence even to the level of Descemet's membrane, demonstrating penetration of the siGLO throughout all layers of the rabbit corneas. These results indicate that topical delivery of siRNAs to corneas wounded by blast injuries can be accomplished rapidly and with essentially no specialized instruments when the siRNAs are complexed with nanocarrier particles. These results will be a great advantage in the next series of experiments when we treat rabbit corneas following excimer laser ablation with the triple combination of siRNAs.

Figure 22. **Nanoparticles deliver siRNA to all layers of the cornea in organ-culture and rabbits.** Whole rabbit globes were ablated to 125 microns using an excimer laser and treated with 3 doses of fluorescently labeled scrambled siRNA complexed with a nanoparticle for 1 minute. a) shows the *ex vivo* organ culture of the excised rabbit corneas. Delivery of fluorescently labeled siRNA to different corneal layers is compared between – b) siRNA alone c) siRNA using nanoparticles d) magnified image of cornea delivered with fluorescently labeled siRNA sequence. The blue color shows the cell nuclei, which were stained with DAPI, and the green shows the fluorescence of the delivered scrambled siRNA.



Objective 2c – To confirm previously reported significant reduction of the downstream mediator of scarring, smooth muscle actin (SMA), by using immunohistochemistry. Also, compare the efficacy of the efficient triple combination to an inefficient triple combination with poor synergism between the siRNA sequences to knockdown collagen.

Low passage number rabbit corneal fibroblast cultures were grown to near confluency in T-75 culture flasks in DMEM supplemented with 10% newborn calf serum (NCS). The cells were harvested with trypsin/EDTA solution and 100 ul of resuspended cell suspension containing ~10^5 were seeded into cells 96 well culture plates in DMEM medium containing 10% NCS. When the cells reached initial confluency, the medium was removed and replaced with serum-free DMEM. After 48 hours of culture, estradiol and TGFB1 was added to the medium. After 24 hours of incubation, a second dose of estradiol and TGFb1 was given to the cells and the cells were transfected with the triple siRNA combination using the optimized transfection reagent and after 6 hours of incubation with the siRNA/transfection reagent, the medium was removed and replaced with DMEM. After a further 24 hours of incubation a third dose of estradiol and TGFB1 was given. After 24 hours, the cells were fixed in 4% paraformaldehyde for 10 minutes. They were then washed in PBS and treated with cold methanol for 15 minutes at -20C. They were washed again in PBS and blocked in goat serum for 1 hour. The samples were incubated with an SMA antibody congujated with Cy3 for 1 hour. The cells were wash again in PBS before mounting them with DAPI. They were imaged using a confocal microscope for taking image.

Figure 23. **The Synergistic triple combination (T1R2C1) is more effective than the Non-synergistic triple combination (T2R1C2) in blocking downstream mediators.** Cultures of Rabbit corneal fibroblasts stimulated with TGFB1 and Estradiol were transfected with synergistic (T1R2C1) and non-synergistic (T2R1C2) triple siRNA combination (90nM) using Mirus transfection reagent. The RNA samples were collected 48 hours after transfection and were analyzed using qRT PCR. a) The RNA level reduction percentages of five genes (target and downstream) are compared between the synergistic (T1R2C1) and the non-synergistic triple combination (T2R1C2). All expression levels were normalized to 18S rRNA. The knockdown percentages were calculated with respect to a scrambled siRNA control. To stain for SMA,

forty-eight hours after transfection, the cells are fixed in 4% paraformaldehyde, blocked in goat serum and then incubated with cy3 labeled SMA antibody. Images of SMA protein immunohistostaining at 20X are shown for: b) synergistic triple combination (T1R2C1), c) non-synergistic triple combination (T2R1C2) and d) cells alone. e) gives the percentage reduction of SMA staining calculated through image analysis from 4 different areas in 3 replicate wells. ($p<0.05$)

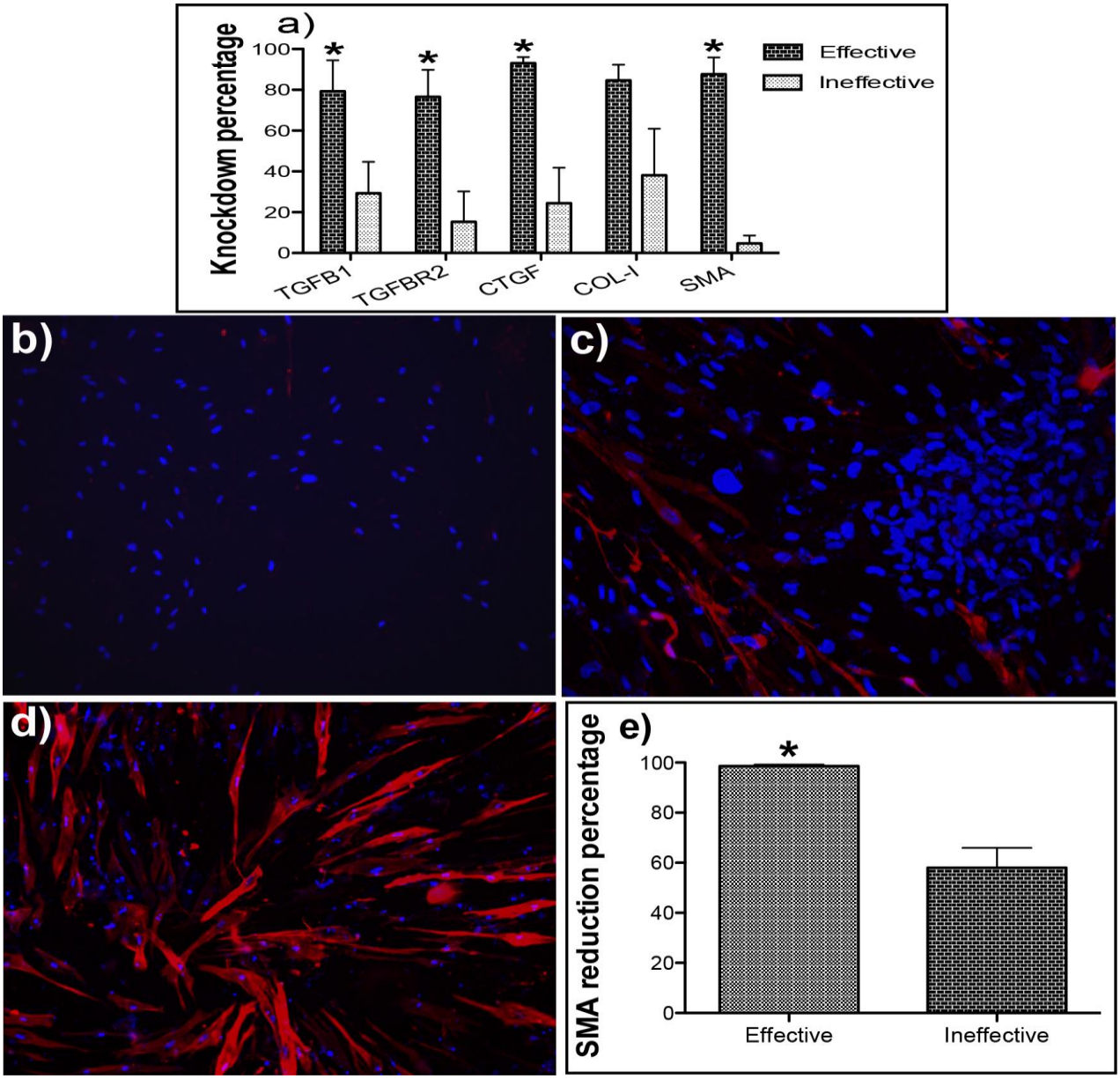
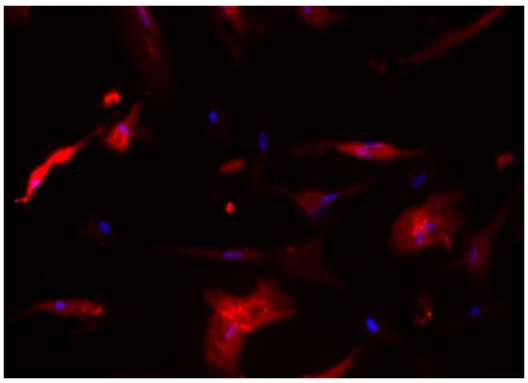


Image analysis procedure
Step1. The images are initially opened using the software ImageJ.

Figure 5. 20x confocal image that was analyzed for levels of alpha smooth muscle actin staining.

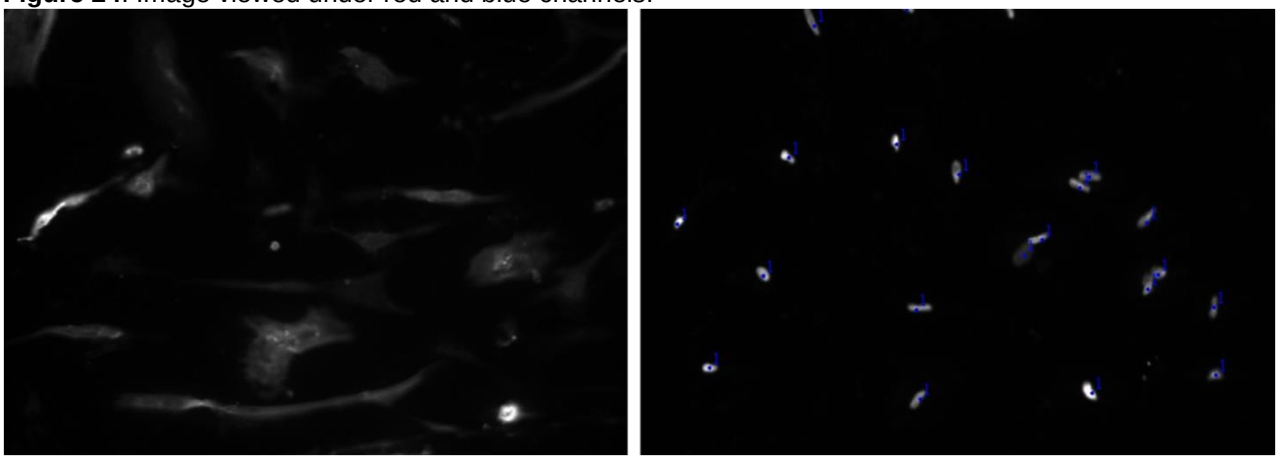
A

B



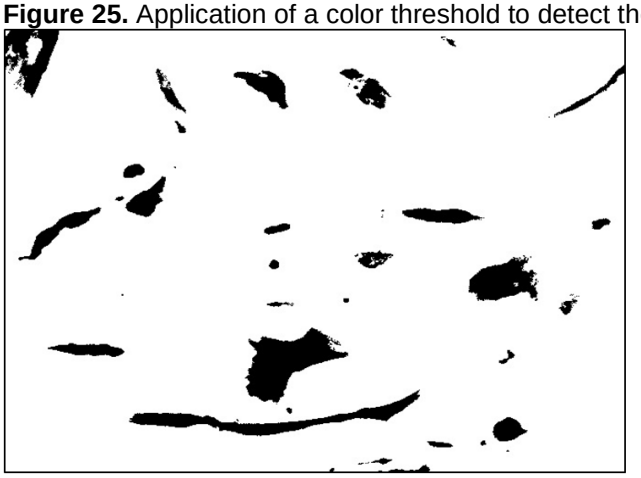
Step2. The image was split into 2 channels – red and blue. SMA was detected by the red channel and the blue channel detected by the DAPI stained nuclei. The number of cells is counted using the cell counter tool.

Figure 24. Image viewed under red and blue channels.



Step3. A color threshold was applied so that only the SMA stained areas are detected. Finally, the percent area stained by SMA was measured and expressed in terms of the number of cells.

Figure 25. Application of a color threshold to detect the SMA stained areas.



	Area Mean	% Area
Total	344064	100
SMA Stained	23,114	6.72

The graph comparing the reduction of SMA in cells treated with synergistic and non-synergistic siRNA triple combination is given in Figure 4-e. It comports with the previously obtained RNA level knockdown measured using qRT-PCR.

Objective 2d– To study the change in expression of CTGF and TGFB1 after excimer ablation in rabbit corneas after ablation at different time points to improve siRNA targeting knowledge.

An excimer laser was used to reproducibly create deep (1/3 the total depth of the cornea), bilateral, PTK ablations (8 mm diameter) to the central cornea of adult NZW rabbits. After ablation, rabbits were euthanized at 2 different time points, day

1 and day 2. A total of 4 rabbits were used for each time point. The cornea was excised and RNA from the different layers (epithelium, stroma and endothelium) were extracted. A cDNA library was generated which was used to probe the expressions of TGFB1 and CTGF. On day 1, the highest expression of CTGF mRNA was in the endothelium, followed by stroma and finally the epithelium. However on Day 2, the stroma had the greatest levels of expression of CTGF. On day1, there was very little expression TGFB1 mRNA throughout all cell layers of the cornea. On Day 2, there was a large increase in the production of TGFB1. The highest expression of TGFB1 mRNA was found in the endothelium followed by stroma and epithelium.

Figure 26. Change in fold CTGF expression at different time points post ablation

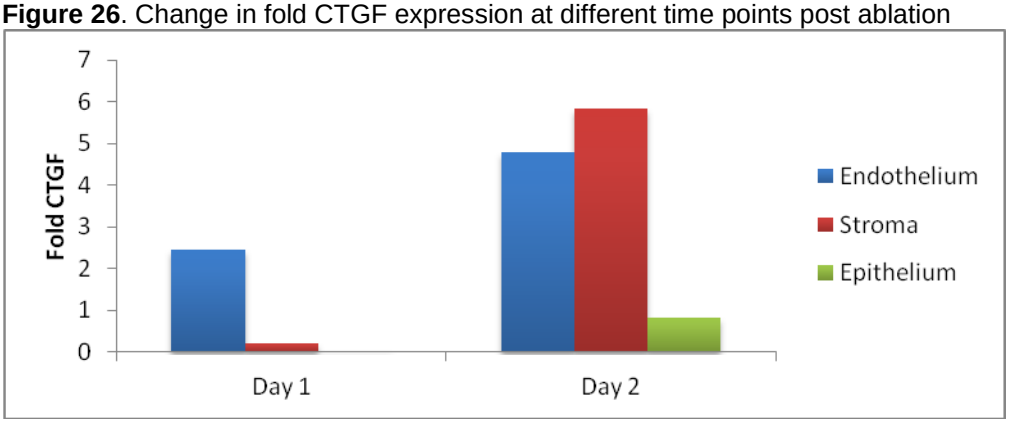
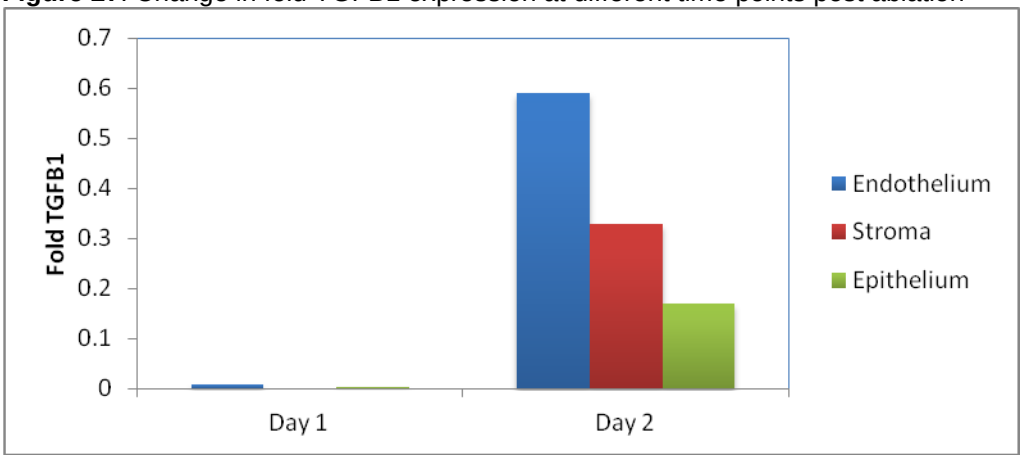


Figure 27. Change in fold TGFB1 expression at different time points post ablation



Objective 2e– To optimize the parameters of the in-vivo confocal microscope using rabbit globes. Commercially available rabbit globes were ordered from Pelfreeze. They were then used to optimize the various parameters of the invivo confocal microscope so that optimized images of the three different layers can be visualized. These settings will be used to analyze of the ability of the triple combination of the siRNAs to reduce haze in rabbit corneas after ablation.

Figure 28. Image of the endothelial layer

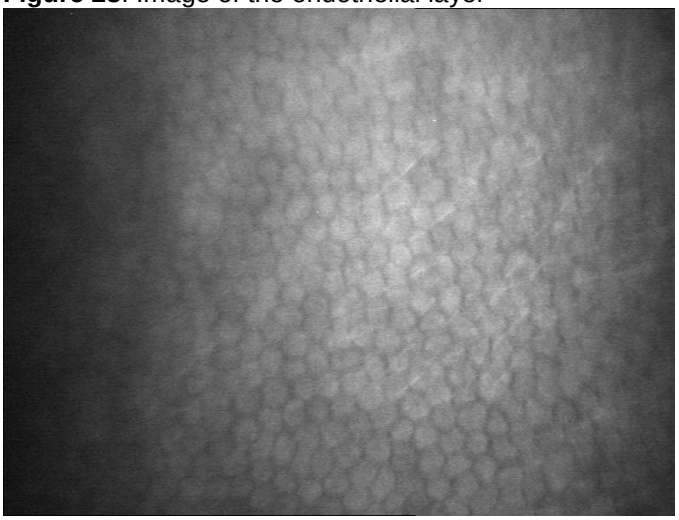


Figure 29. Image of the stromal layer

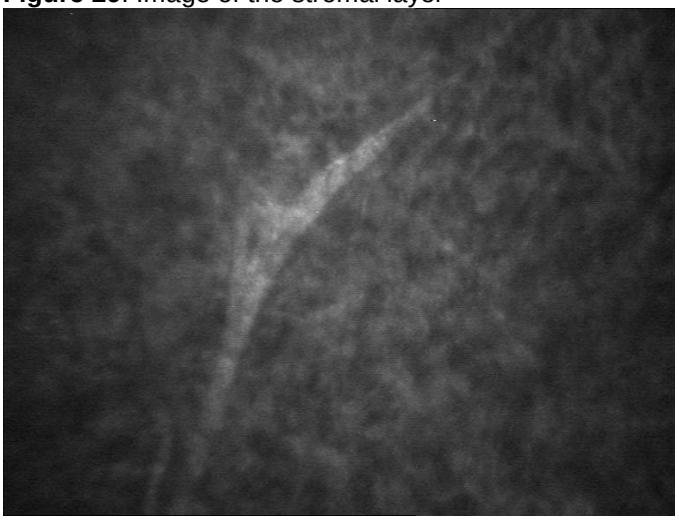
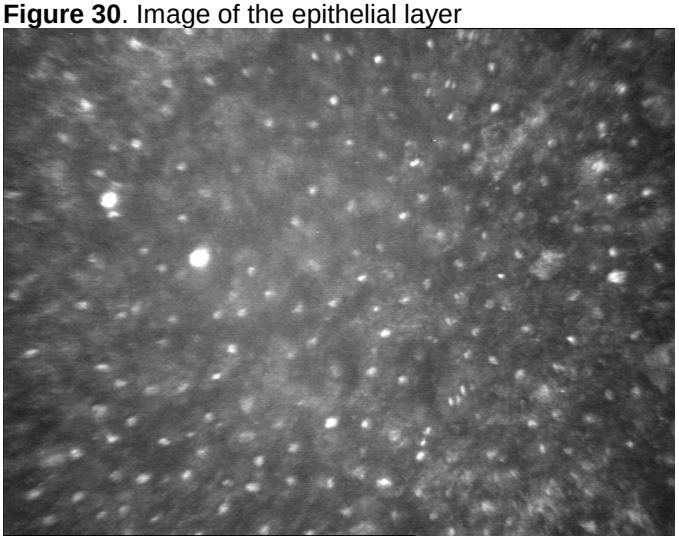


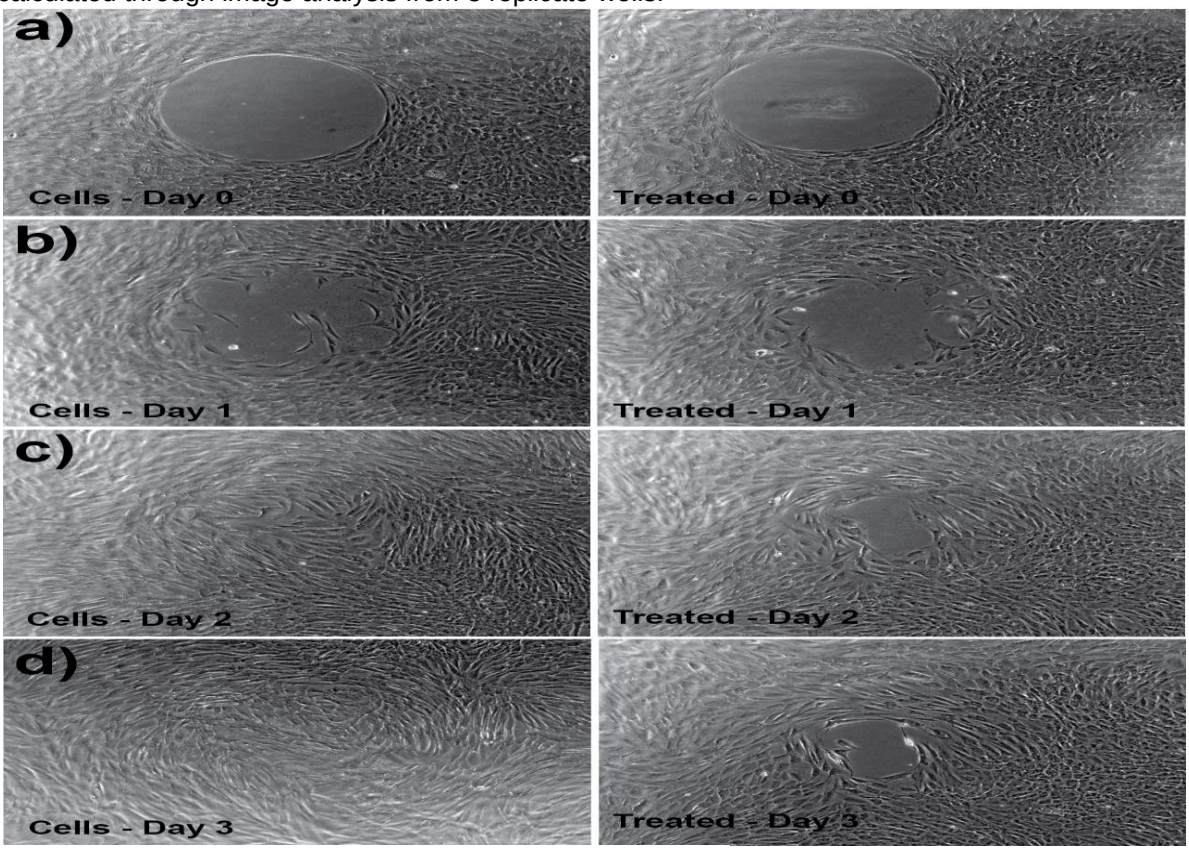
Figure 30. Image of the epithelial layer

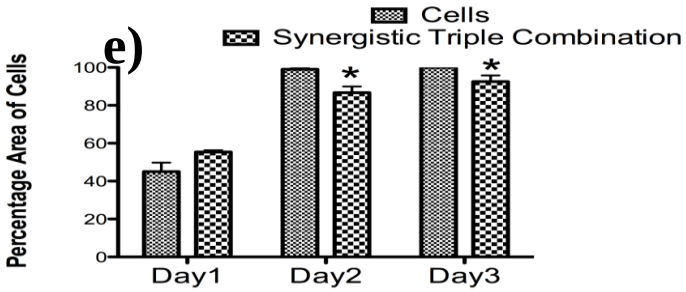


Objective 2f – To evaluate the ability of the synergistic siRNA triple combination to retard the migration of corneal fibroblasts cells in culture

Cultures of rabbit corneal fibroblasts (RbCF) were established by outgrowth from rabbit whole eyes, as described previously (Woost, Jumblatt, Eiferman, & Schultz, 1992). Briefly, epithelial and endothelial cells were removed from corneas, the stroma was cut into cubes of approximately 1 mm^3, placed in culture medium consisting of equal parts Dulbecco's Modified Eagle Medium (DMEM), with 4.5g/L Glucose and 1g/L L-glutamine. Medium was supplemented with 10% heat-inactivated normal calf serum and 1× antibiotic-antimycotic (Gibco BRL). Cell from cultures between passages 2 and 5 were used for all experiments. To increase the expression of TGFB1, TGFBR2 and CTGF, RbCF were place in serum-free media for 48 hours, then the medium was replaced by 8ug/ml of estradiol (Sigma, St. Louis, MO) and TGF-B1 (R&D Systems, Minneapolis, MN) in DMEM. The cells were dosed twice with the estradiol before transfection of the siRNA (Takahashi et al., 1994; Wira, 2002). For the migration assay, RadiusTM Cell Migration Plate (Cell biolabs, San Diego, CA) was procured and used according to manufacturer's protocol. Images were taken at 10x magnification and analyzed using Adobe Photoshop.

Figure 31. For the migration assay, a circular region in the middle of the well was removed using a gel removal solution provided with the Radius® cell migration kit. Panels a) – d) show migration assay results comparing cells alone and synergistic triple combination (T1R2C1) treatment wells. e) gives the percentage area of cells from the migration assays calculated through image analysis from 3 replicate wells.





Apart from reducing the above downstream mediators, the synergistic triple combination (T1R2C1) was also able to significantly inhibit the migration of cells within two days post transfection. The percentage area of cells which was calculated digitally from the three replicates was used as a measure of migration (Figure 31-e)

Objective 2g. Develop an ex vivo model of corneal scarring using organ culture of viable rabbit corneas and assess the effects of the triple siRNA-NP following excimer laser ablation.

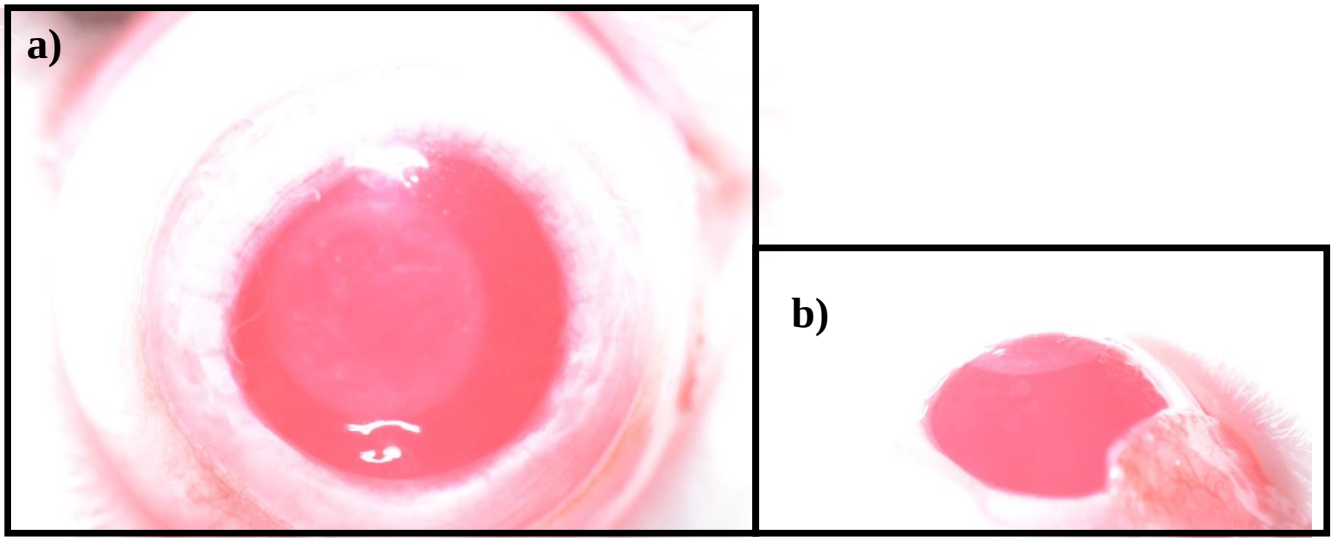
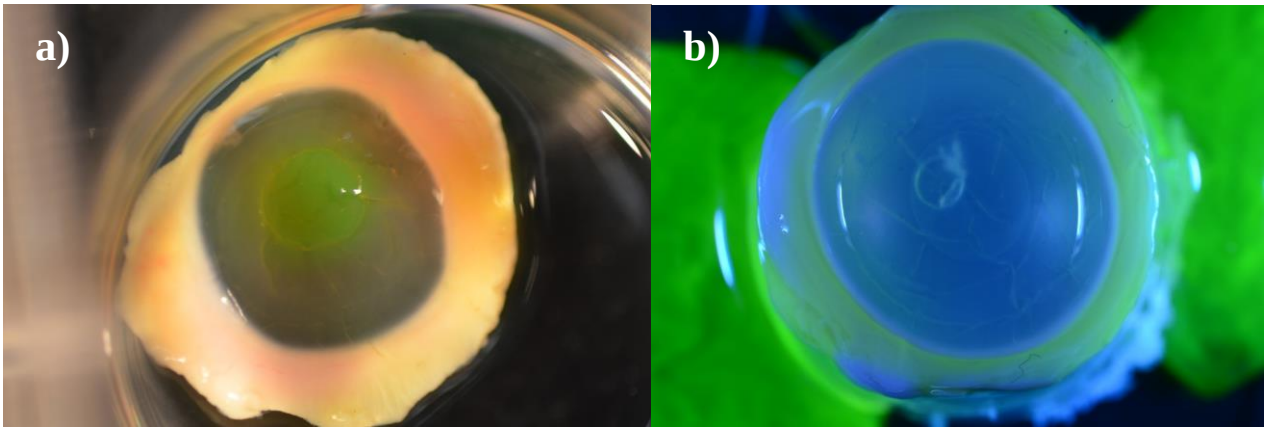


Figure 32. Pictures of rabbit eye 15 days post ablation. a) and b) show clear and consistent haze in the ablation zone after 15 days.

One of the main challenges in the use of siRNA has been the delivery. Corneal drug delivery experiments require sacrification of animals at each time point in the drug concentration profile, and the use of organ culture therefore would help reduce our need for animal experiments. Studies have also shown eunucleated rabbit eyes sustained in culture retain wound healing dynamics and are comparable to the live rabbit eye model in terms of laser radiation exposure. Hence, in order to make the most efficient use of animals, we followed the exact rabbit ablation procedure on excised corneal globes and monitored it for a period of 2 weeks for the presence of corneal haze.

Objective 2h – To recreate the in vivo scar formation in ex vivo corneal culture

Corneas were organ-cultured as previously described in (Castro-Combs et al., 2008; Chuck et al., 2001; Richard, Anderson, Weiss, & Binder, n.d.). Briefly, using a jigsaw, the ends were cut from laboratory test tubes at the indicator line nearest the bottom. The cut edges of the domes were coated with super glue and aseptically placed firmly concave down into the center of each well of a 12-well tissue culture plate. The plates were then sterilized by placing them in UV overnight. Fresh rabbit globes from Pelfreeze were ablated to 155 microns using an excimer laser as described above. Corneal rims were then surgically extracted from the rabbit globes and using sterile forceps, grasping only the scleral rims and not cornea; each rim was placed over a single dome. Each well was filled with supplemented DMEM containing 10% fetal bovine serum (Gibco, BRL, Life Technologies). Incubations were carried out in 5% CO_2 and 95% air at 37C for the duration of the experiment. The media was changed every 2 days. The corneas were photographed and monitored for the next two weeks.



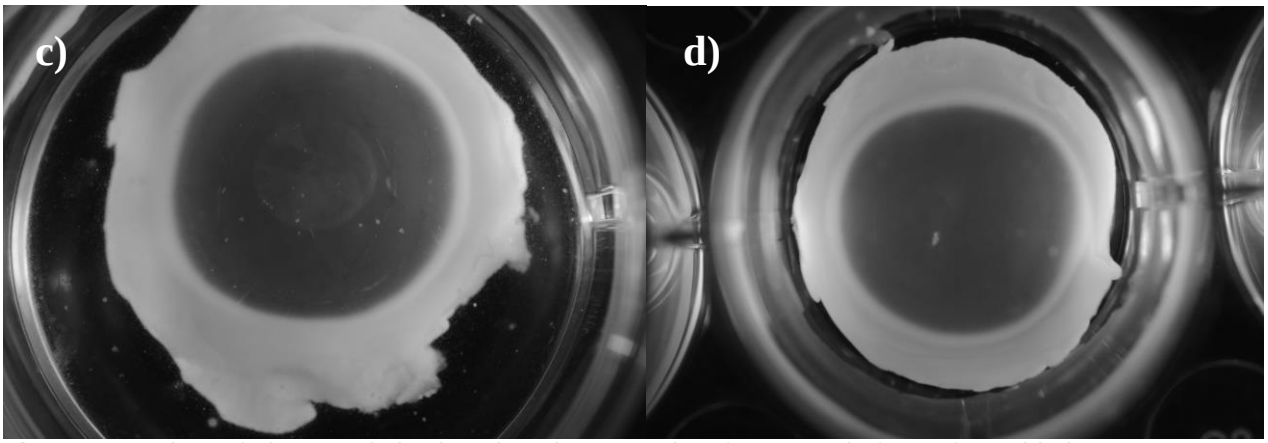


Figure 33. A drop of Fluorescein is placed on the corneal surface to stain for surface ablations. Corneas were stained and photographed immediately after ablation (Image a) to show the ablation zone. The corneas were then stained after 15 days. No visible green color is present on the surface indicating re-epithelialization. The corneas start developing haze 15 days post ablation (Image c) when compared with the unablated cornea control (Image d).

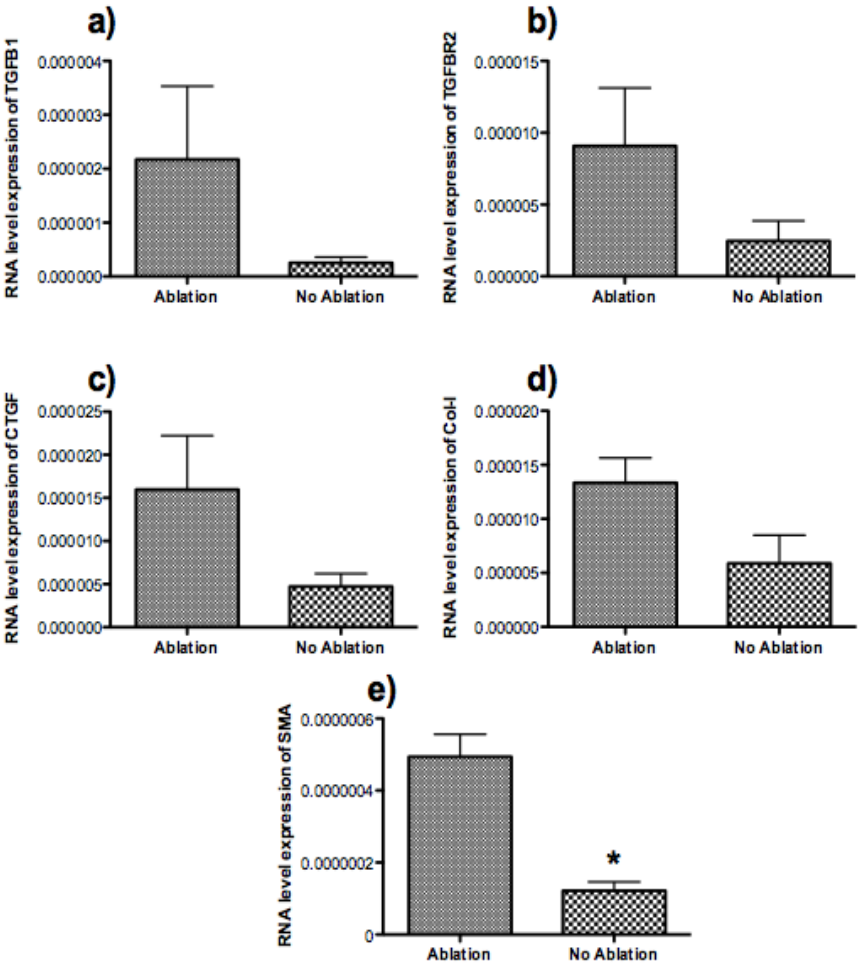


Figure 34. An 8mm biopsy punch was used to obtain the central scar-like region of the corneas and the expression levels of 5 profibrotic genes were analyzed with qRT-PCR. All expression levels were normalized to 18S rRNA. The expression levels of all the profibrotic genes were higher when compared to the unablated corneas. In particular, the expression of SMA, a key indicator of scar tissues, in the ablated corneas was significantly higher than that of unablated corneas.

Objective 2i – To test the efficacy of the ex vivo corneal culture as a therapeutic model

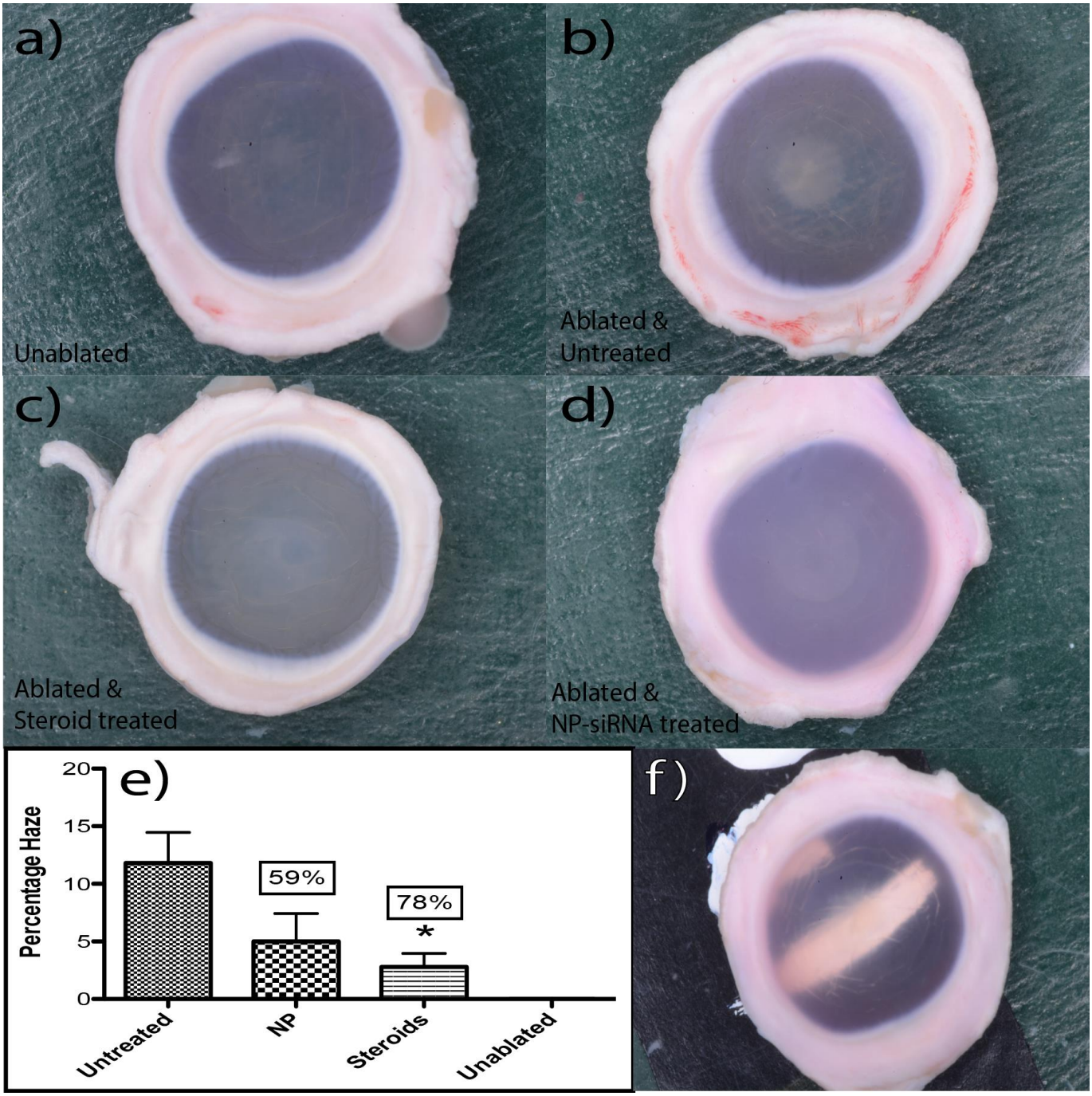
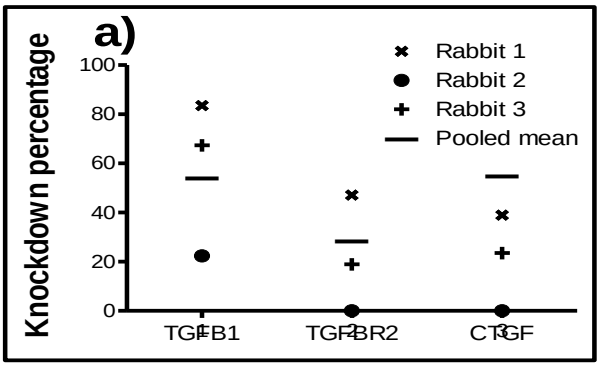


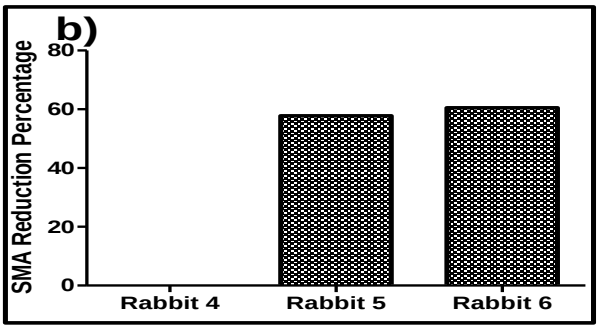
Figure 35. Freshly obtained rabbit globes were ablated to a depth of 155um and cultured at the air-liquid interface for 21 days. In this study, the effect of steroids and the triple siRNA combination delivered using nanoparticles in reducing corneal scarring were assessed. The corneas were cultured in serum free media supplemented with 1%P/S and 1ng/ml of TGFB1. There was a remarkable reduction in haze formation in the both the treatment groups as show in images a) – d). The scar formation in these images was numerically quantified by measuring the reduction in pixel intensity of a white line which was placed under the cornea. The results of the image analysis show that steroids were effective in significantly reducing the haze formation by 78% when compared to the untreated control The triple siRNA combination was also effective in reducing corneal haze formation by 59%.

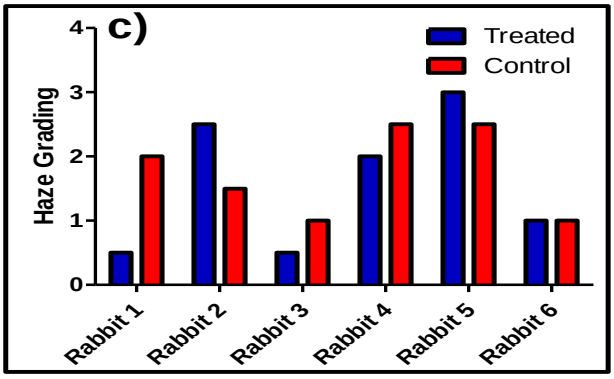
Objective 3a - Observe and evaluate the effects of a topical application of the previously optimized siRNA-nanocarrier complex post wounding in a rabbit model

Adult New Zealand Rabbits free of disease were used and treated according to ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Excimer ablation and collection of corneas was performed as previously described (Netto et al., 2006). Briefly, rabbits were anesthetized with isoflurane inhalation, and proparacaine eye drops provided topical anesthesia. Laser ablations were performed to both eyes of each rabbit with a Summit SVS eximer laser that is committed to animal vision research. Using the laser in phototherapeutic keratectomy mode, the central 6 mm diameter area of the cornea was be ablated at a dose of 160mJ/cm2 to an initial depth of 80 microns to remove the epithelium and then the final 45 microns were ablated by placing a mesh over the cornea to make an uneven ablation. The eyes were pretreated with 50uM EDTA for 10 minutes. A total of 150ul of the nanoparticle complexed with the siRNA triple combination was added to the right eye while the left eye was treated with the vehicle control and was considered as a paired negative control. The eyes were held open for 3 minutes to allow the nanoparticle to penetrate the stroma before being disturbed. No postoperative topical steroid was used to ensure that the wound healing process is not altered with anti-inflammatory agents. One and fifteen days after the treatment, corneas were collected, homogenized in a pestle with liquid nitrogen and then transferred to TRIzol. The RNA was then extracted using a hybrid RNA extraction protocol with RNeasy spin columns (Rodriguez-Lanetty, 2007).

Figure 36 - For the in vivo experiment, the corneas of 6 rabbits were unevenly ablated to 125 microns using an excimer laser. The right eye was treated with150uL of the synergistic triple combination (T1R2C1) complexed with nanoparticles and the left eye received the vehicle control. Three rabbits for each time point were sacrificed and RNA was extracted for analysis by qRT PCR at 1 and 14 days post siRNA treatment. a) gives the RNA level knockdown percentages of the target growth factors at day1 while b) gives the RNA level knockdown percentage of SMA at day15. All expressions were normalized to 18S rRNA and knockdown percentages were calculated with respect to the left eye. The corneal scarring was graded by an ophthalmologist on a scale from 0-4. The scores for both the treated and control eyes are plotted for each rabbit in c)







In the day1 samples, the synergistic triple combination (T1R2C1) gave an average of knockdown of 57% for TGFB1, 25 % for TGFBR2 and 24% for CTGF (Figure1-a). One of the rabbits (rabbit 1) had a maximum knockdown of 80% for TGFB1, 57% for TGFBR2 and 46% for CTGF indicating some the siRNA combination was effectively delivered to the corneal stroma in this animal. In the day15 samples, the synergistic triple combination (T1R2C1) gave an average knockdown of ~40% in the RNA level expression of SMA. Two of the three rabbits show a ~60% reduction in the expression of SMA demonstrating a reduction in the RNA levels of SMA by treatment with synergistic triple combination (T1R2C1) (Figure1-b). All knockdown percentages were calculated with respect to the left eye, which received vehicle control without the siRNA. The haze grading of the rabbits also showed a positive trend. Three out of the six rabbits had a lower haze grading score after a period of two weeks (Figure 36-c).

SUMMARY OF KEY RESEARCH ACCOMPLISHMENTS

- Optimal siRNAs were identified for each of the three key target genes, TGFb, TGFbRII, and CTGF that produced at least 70% knock down of target mRNAs and similar knock down of the target proteins
- Optimal double combinations of siRNAs were identified that produced greater than 80% knock down of at least one of the three target gene mRNAs
- An optimal triple combination of siRNAs was identified that produced 97% knock down of mRNA for type I collagen mRNA in RCF cultures
- A formulation consisting of NP-siRNAs was developed and produced extensive transduction of rabbit corneas in vitro
- A photographic method was developed that quantitatively measured levels of light scattering (haze) in rabbit corneas
- An advanced ex vivo model of viable rabbit corneas was developed that generated increases in key target gene mRNAs and proteins and also developed extensive haze
- The excimer laser ablation model for rabbit corneas was optimized along with a quantitative image analysis system to measure levels of corneal scarring as indicated by light scattering, which is clinically called corneal haze
- Treatment of both the *ex vivo* and *in vivo* models of rabbit corneal scarring with the NP-triple siRNA showed reduction of target mRNAs, which produced clinically significant reductions in corneal scarring as measured by both light scattering, that is visualized clinically as corneal haze.

REPORTABLE OUTCOMES

The results of these experiments have been submitted in two major manuscripts and as four abstracts at the 2012 and 2013 annual meeting of the Association for Research in Vision and Ophthalmology and at the 2012 annual meeting of the Wound Healing Society.

CONCLUSIONS

The results of the experiments conducted during the two years of this research project have achieved an important milestone, which is the identification of a triple combination of siRNAs targeting three key genes (TGFb, TGFbRII, and CTGF) that synergistically reduces the level of mRNAs for type I collagen gene and aSMA by >95% without causing any cell toxicity. This triple combination hold great promise for a new drug formulation that will reduce corneal scarring without significant detrimental side effects that are produced by other drugs like mitomycin-C, 5-fluoruracil or steroids that are used in desperation to reduce corneal scarring.

REFERENCE LIST

1. G. Schultz *et al.*, *Eye* **8 (Pt 2)**, 184 (1994).

2. G. Schultz, N. Chegini, M. Grant, P. Khaw, S. MacKay, *Acta Ophthalmol. Suppl* 60 (1992).

3. S. Tuli, M. Goldstein, G. S. Schultz, in *Cornea*, J. H. Krachmer, J. M. Mannis, E. J. Holland, Eds. (Elsevier Mosby, Philadelphia, 2005) ,chap. 9, pp. 133-150.

4. D. Grimm, *Adv. Drug Deliv. Rev.* **61**, 672 (2009).

5. T. D. Blalock *et al.*, *Invest Ophthalmol. Vis. Sci.* **44**, 1879 (2003).

APPENDICES

NONE